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DYNAMICS AND BIOAVAILABILITY OF FOLATES IN BOVINE LIVER AS A FUNCTION OF PROCESSING AND FROZEN STORAGE

A Dissertation
Submitted to the Graduate Faculty of the
Louisiana State University and Agricultural
and Mechanical College in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
in
The Department of Food Science

by
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August, 1986

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FADI MICHAEL ARAMOUNI

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DEDICATION

This work is dedicated to the two persons whose immense sacrifices and inexhaustible love made all the difference in my life:

My Father Michael
My Mother Violet

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TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

LIST OF FIGURES

LIST OF FIGURES (continued)

LIST OF FIGURES (continued)

LIST OF TABLES

LIST OF TABLES (continued)

ABSTRACT

Raw, broiled and fried beef liver samples were stored at -20°C in either vacuum or air-saturated packages. At periods of 30, 60 and 90 days the folates were extracted and analyzed by reverse phase high performance liquid chromatography (HPLC). Tetrahydrofolic acid (THF), dihydrofolic acid (DHF), 5-CH_3-THF, 5-CHO-THF and pteroylglutamic acid (PGA) were identified and quantitated. Two major folate oxidation products, pterin-6-carboxylic acid (pt-6-COOH) and p-aminobenzoylglutamic acid (p-ABG) were also detected. The total folate content of beef liver was higher than previously reported. PGA was not found in any of the samples but a large peak corresponding to the retention time of DHF was observed. The order of stability of the folates towards cooking was: DHF < 5-CH_3-THF < 5-CHO-THF < THF. The stability of THF may have been a function of the reduction of DHF. Levels of 5-CH_3-THF, THF and DHF decreased during frozen storage while those of 5-CHO-THF generally increased. These trends were not always linear over time. Folate interconversions and deconjugation of pteroylpolyglutamates during storage was suspected. The HPLC quantitation of total folates consistently resulted in higher values than that by the Lactobacillus casei microbiological assays.

CHAPTER I

INTRODUCTION

Although a widespread borderline folate deficiency is presently recognized in various parts of the world, current data on folacin activity are lacking. Dietary studies on folates have been scarce because of the difficulties encountered while assessing the folate content of foods (Swee et al., 1984). Since folate occurs naturally as a series of pteroylglutamate compounds of different activity, microbiological estimates may vary widely according to the conditions of the assay (Stokstad et al., 1977). Alternative methods of folate determination, whether chemical, colorimetric, enzymatic or fluorometric are not particularly suitable for biological material (Day and Gregory, 1981).

The reliability of data collected on folacin activity in foods by the traditional assay methods have been the subject of controversy (Bates et al., 1982; Smithells, 1984). The limited amount of information available on the dynamics of folates has been performed in aqueous buffers (O'Broin et al., 1975) or, at best, in liquid model systems (Ristow et al., 1982). In as much as this paucity of information is currently acknowledged, there is a need to measure folate dynamics in natural food systems and to relate those dynamics to biological activity. Beef liver

was chosen for this study due to its high folate content and to the presence of most, if not all, folate enzyme systems. Three main objectives were identified:

1. To determine the predominant folacin derivatives in bovine liver and their main oxidation products by high performance liquid chromatography;

2. To study the dynamics of folate behavior in bovine liver as a function of conventional preparation methods and duration of storage;

3. To correlate the dynamic behavior of folates to their availability as estimated by the Lactobacillus casei microbiological method.

CHAPTER II

LITERATURE REVIEW

Historical Aspects

In the early 1930's, Dr. Lucy Wills observed that Indian women with megaloblastic anemia responded to a factor found in yeast supplements. A similar effect was also reported in monkeys whose diet, resembling that of the Indian women, consisted mainly of white bread and rice. Later experiments showed that the Wills factor was similar to Vitamin M which protected against cytopenia in monkeys, and Vitamin B_6 which protected against anemia in chicks. Also, nutritional similarity was established for Factor U, a growth factor for chicks, the Lactobacillus casei factor and the Leuconostoc citrovorum (now Pediococcus cervisiae) factor. The name folic acid, derived from the latin word for leaf, was first proposed by Mitchell and Williams in 1941 to indicate yet another similar compound isolated from spinach leaves and necessary for the growth of Streptococcus faecalis R. (Pike and Brown, 1975, p. 116). In later years, the determination of the structure of pteroylglutamic acid revealed that all of the above mentioned factors were different forms of the same vitamin. Currently the term folacin is used to include folic acid and all its derivatives. Subsequently, upon isolation and purification, the vitamin was found to consist of a

pteridine nucleus, p-aminobenzoic acid and one or more glutamic acid residues. Both p-aminobenzoic acid (PABA) and pteroylglutamic acid (PGA) were first believed to possess vitamin activity. However, it now appears that whereas PABA can be used by certain bacteria to synthesize the larger molecule, most mammals require PGA as the vitamin.

Nomenclature and Derivatives

Tannenbaum et al. (1985) reported that about 140 different folate derivatives could exist if the number of glutamic acid residues is limited to six. Out of those, only 35 have been isolated. The task of naming all derivatives is formidable. An advisory panel made the following recommendations concerning folate nomenclature to the Commissions on Nomenclature of IUB and IUPAC (Blakley and Benkovic, 1984, p. xi-xiv).

Definitions. Folates are heterocyclic compounds based on 4-[{(2-amino-4 (3H)-oxopteridin6-yl)methyl}amino] benzoic acid, commonly known as pteroic acid. Their salts and acyl groups are named pteorates and pteroyls, respectively.

Numbering. Numbering of the atoms is defined as indicated in the structural formula (Fig. 1).
When indicating substitution on nitrogen atoms 5, 8 and 10, it is necessary to introduce the letter N.

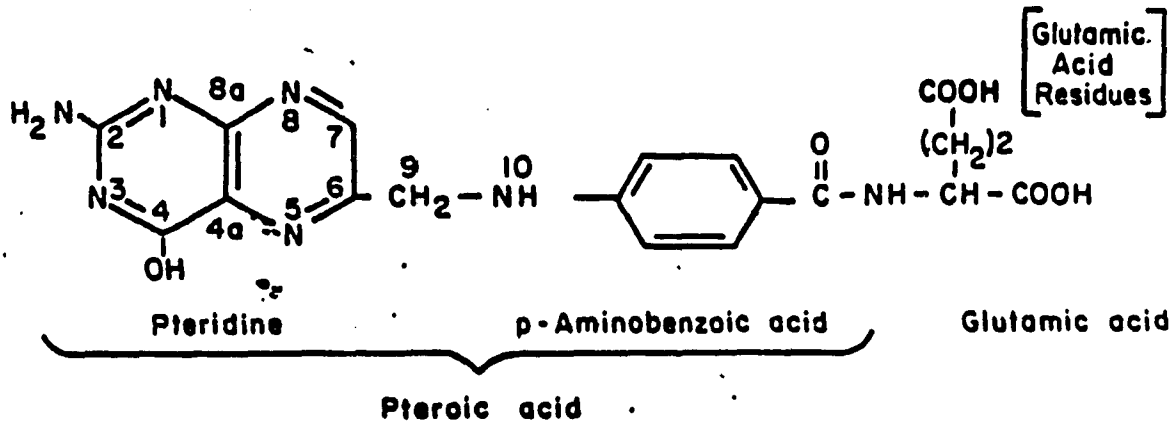


Fig. 1--Structure of folic acid

Glutamic acid conjugates. When pteroic acid is conjugated with glutamic acid residues, the resulting compounds are called pteroyldiglutamic acid, pteroyltriglutamic acid and so on. Those terms may be used in a generic sense irrespective of the state of reduction of the pteridine ring. This is also true of the term folates, which may designate any member or mixture of members of the pteroylglutamates family. With the substitution of one molecule of glutamic acid, the term pteroylmonoglutamate is not to be used and the terms folate and folic acid are preferred to pteroylglutamate and pteroylglutamic acid, respectively.

Reduced compounds. The prefixes dihydro-, tetrahydro- and so on, with corresponding numerals, indicate reduced

folate compounds. Tetrahydro- and dihydro- folates are assumed to be 5, 6, 7, 8 and 7, 8, respectively. Whenever possible, appropriate designation of natural stereoisomers should be indicated.

Substituents. The substituent prefixes, taken from the Organic Nomenclature Rules, refer to one substituted hydrogen atom in the case of formyl, methyl or formimino, and to two substituted hydrogen atoms in the case of methylene and methenyl. Conflicting with the general rule, the alphabetical order is disregarded in the nomenclature of reduced substituents and the prefix indicating reduction occurs immediately before the stem name (e.g., dihydrofolate, tetrahydropteroyldiglutamate).

Symbols and abbreviations. The following principles are to be applied for brevity and convenience:

1. The term folate is not to be abbreviated and its use is preferred to that of folic acid.

2. Pte is an abbreviation of pteroate, pteroyl- or pteroic acid.

3. The symbol Glu indicates L-glutamate linked to other glutamate molecules by amide bonds through the -carboxyl group.

4. The number of glutamate residues is indicated by subscript numerals. Thus, pteroylglutamates are abbreviated by such symbols as $PteGlu_3$, $PteGlu_5$ and so on.

5. Reduced derivatives are indicated by H_2 or H_4 which may be used with "folates" or "pteroylglutamates".

6. Substituents are abbreviated according to the following list:

Formimino	(Formimidyl)	NHCH
Formyl		HCO-
Methyl		CH_3-
Methylene		-CH_2
Methenyl	(Metheno)	-CH-

Although no official action has yet been taken on the above recommendations, they have already been adopted in recent publications.

Physical and Chemical Characteristics

Stability and solubility. Crystalline folic acid is a yellow dull substance with a molecular weight of 441.4 and an empirical formula of $C_{19}H_{19}N_7O_6$. It does not have a boiling point as such but chars at 250°C. It is only slightly soluble in water and organic solvents, but its solubility increases with temperature and aqueous acids or bases. Solutions of folic acid or its salts are unstable to light and oxidizing agents. Moreover, studies triggered by the increased interest in food irradiation have shown an extensive degradation of folic acid in water at room temperature when subjected to gamma radiation (^{60}Co) (Kishore et al., 1978).

O'Broin et al. (1975) studied the nutritional stability of various naturally occurring monoglutamate derivatives of folic acid. PGA and 5-formyl-tetrahydrofolate (5-CHO-THF) were found to be nutritionally stable for weeks. 5-methyl-tetrahydrofolate (5-CH_3-THF) was unstable under acidic conditions but quite stable at pH values above neutrality. Unsubstituted tetrahydrofolate

(THF) was the least stable, especially under basic conditions, while 10-formyl-tetrahydrofolate (10-CHO-THF) was surprisingly stable. This was probably due to its conversion to the 5-formyl derivative. All compounds studied had increased nutritional stability in the presence of ascorbate which was a superior protective agent for folates when compared to 2-mercaptoethanol. Paine-Wilson and Chen (1979) related the lability of folate derivatives in a buffered solution to the nature of the buffer and final pH. Solutions of folic acid and 5-CHO-THF were stable up to 10 hours at 100°C over the pH range 4-12 while THF in solution showed 90% degradation in 4 hours. Maruyama et al. (1978) investigated various aspects of stability and oxidative cleavage of folates. The products generated by alkaline permanganate ($KMnO_4$) oxidation were evaluated for several folic acid derivatives as summarized in Table 1.

Acidic dissociation constants. Acidic dissociation constants are important considerations in analytical work aimed at the separation of folate derivatives. Cocco et al. (1981) reported pK' values of 2.40 and 8.25 at the 1-N and 3,4-NHCO positions of folate, respectively. An approximate 1 unit decrease in pK' values for dihydrofolate (DHF) and THF have been reported relative to the 1-N position in folate, while at 3,4-NHCO the increase in pK' was about 1 Unit for DHF and 2 units for THF. The pK'

Table 1. Oxidative cleavages of folates

Compound	Stability in acid (pH 1.0)	Stability in base (pH 13.0)	Product after $KMnO_4$ oxidation
Folic acid	S	S	p-ABG
DHF	RS	U	p-ABG
THF	U	U	p-ABG
5-HCO-THF	CT 5,10=CH-THF	S	p-ABG
5,10=CH-THF	S	CT 10-HCO-THF	10-HCO-Folate
5,10-DHF	U	RS	10-HCO-Folate
10-HCO-THF	CT 5,10=CH-THF	RS	10-HCO-Folate
10-HCO-Folate	S	CT Folate	10-HCO-Folate
5-CH3-THF	RS	--	5-CH3-DHF
5-CH3-DHF	VL (p-ABG)	S	5-CH3-DHF
10-HCO-$Pteglu_7$	S	CT $Pteglu_7$	10-HCO-$Pteglu_7$
p-ABG	S	S	p-ABG & Unk.

S = Stable
U = Unstable
RS = Relatively stable
CT = Converts to
VL = Very labile
Fol. = Folate
Unk. = Unknown

values at the α-COOH, γ-COOH and 3,4-NHCO positions of 5-CHO-THF were 3.1, 4.8 and 10.4, respectively. All dissociation constants were determined by comparison to model compounds.

Absorption spectra. Spectrophotometry has been used for the quantitation of folates (Franke and Rothe, 1975). Folic acid and its analogues are known to absorb both in

the visible and U.V. range producing up to four bands, depending upon the degree of ionization. Reduced folates show one or two absorption bands in the 250-300 nm range. The max. values for folate derivatives of interest in the present study are presented in Table 2.

Table 2. λ max. values of folate derivatives

Compound	pH	max. (nm)
Folic acid	1.0	296
	7.0	282, 350
DHF	6.8	282
THF	7.85	296
10-HCO-THF	7.5	260
5-HCO-THF	7.0	287
5-CH_3-THF	1.0	270, 294
	7.0	290

Compiled in part from Blakley and Benkovic (1984, pp. 78-80)

Folate Biosynthesis

Early work indicated that pterin precursors in bacterial preparations produced folate like compounds upon incubation with p-aminobenzoic acid. In later experiments, 6-CH_2OH-7,8-H_2-pterin was found to be the most active precursor of folate biosynthesis in Lactobacillus plantarum, Escherichia coli and pea seedlings. The reaction necessitates the presence of p-aminobenzoate, ATP and magnesium ions. The products derived from p-

aminobenzoate and p-aminobenzoylglutamate are 7,8-H_2-pteroate and 7,8-DHF. Only one enzyme seems to be involved since the utilization of p-aminobenzoate was found to be competitively inhibited by glutamate (Ortiz, 1970). A pathway for the formation of the pteridine ring in folic acid from guanadine triphosphate (GTP) was proposed by Mitsuda and Suzuki (1968). The pathway involves cleavage of the imidazole portion of GTP with removal of carbon 8, an Amadori rearrangement of the ribose residue and finally the cyclization of the compound prior to its involvement in the synthesis of xanthopterin. Later work by Brown and Williamson (1982) confirmed that GTP is the purine compound used in the biosynthesis of all 2-amino-4-hydropteridines, including the pteridine ring of folic acid. The biosynthetic pathway to DHF from pterin precursors was reviewed by Shiota (1984) and is shown in Figure 2.

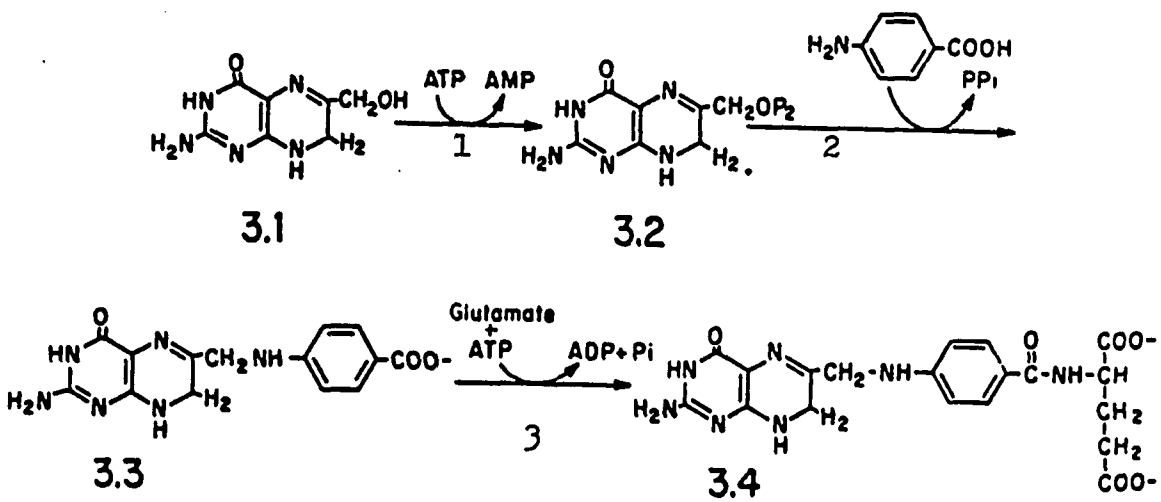


Fig. 2--Biosynthetic pathway of DHF

The endproduct, DHF, can be reduced to THF through the action of the enzyme dihydrofolate reductase according to the following reaction:

DHF ----------> THF

NADPH + H^+ ⟶ NADP

Finally, some reports have indicated that folate synthesis might be influenced by biotin. The metabolic interrelationship between the two vitamins was reported both in E. coli and in rats by Nair and Noronha (1974).

Inhibitors of Folate Biosynthesis

The enzyme, dihydrofolate, synthetase that catalyses reaction 3 (Fig. 2) requires the presence of a monovalent and a divalent cation. It is inhibited by either of its products: adenine diphosphate (ADP) or 7,8-DHF. On the other hand, sulfonamides have long been known to irreversibly inhibit the enzyme dihydropteroate synthase. This is due to their action as alternate substrates for the enzyme (Swedberg et al., 1979) and there is a strong indication that when used as drugs they competitively inhibit p-aminobenzoate utilization. This property of sulfonamides and diphenylsulphones is used effectively in the chemotherapy of various diseases. Dapsone, is one such agent successfully adopted for the treatment of Hansen's Disease (leprosy) in man. The enzyme has been inhibited through the introduction of analogues of 7,8-H2-pteroate

such as 7,8-H2-homopteroate or 7,8-H2-10-thiopteroate.

Dihydrofolate reductase has received considerable attention due to its role in DNA synthesis. The methylation of the uracil moeity of deoxyuridylic acid to produce deoxythymidylic acid is catalyzed by thymidylate synthase. The enzyme requires the participation of 5,10-methylenetetrahydrofolate as a coenzyme which supplies the methyl group, and reappears at the end of the reaction as dihydrofolate. The formation of thymidylate and consequently DNA is sensitive to low levels of THF. The latter can be regenerated from DHF by the action of the enzyme dihydrofolate reductase. Antifolate drugs, such as aminopterin and amethopterin, competitively inhibit this enzyme because of their structural resemblance to DHF. They have been used with relative success to slow the growth of some type of cancers, particularly leukemia (Lehninger, 1978, p. 739).

PteroylPolyglutamates

Occurrence and function. The concept that pteroylpolyglutamates are only storage forms of folic acid has recently been challenged by strong evidence suggesting that folate polyglutamates are the naturally occurring coenzymes for folate mediated reactions in mammalian cells (Hoffbrand et al., 1977). Although folates circulate in the plasma in the monoglutamate forms, about 90% of the folates in mammalian cells exist as polyglutamates and have

been shown to be more active than their monoglutamate counterparts.

The activity of polyglutamates has been established in both folate mediated reactions carried out in vitro and in reactions where they act as substrates for the reducing enzyme dihydrofolate reductase (Coward et al., 1974). In this respect, an important study performed by McBurney and Whitmore (1974) indicated that mammalian cells which were genetically unable to synthesize folate polyglutamates, required extra thymidine, adenosine and glycine in their culture medium. Since the de novo synthesis of all the above compounds is known to require folate coenzymes, it is clear that the polyglutamates, rather than the monoglutamates, are the active folate coenzymes.

The exact mechanism by which the peptidyl chain length affects the regulatory action of the vitamin is still unknown. However, three possible regulatory processes have been considered: a direct effect on the enzyme-coenzyme specificity, an activating and/or allosteric inhibitory effect on the enzymes involved in one carbon metabolism and finally, an alteration of the biological membrane permeability to the folate coenzymes.

The quantity of research compiled on pteroyl-polyglutamates is a reflection of the uncertainties surrounding their presence in food and their metabolic function. Pteroylpolyglutamates are the major form of

folates naturally occurring in foods. The effects of their presence in terms of the bioavailability of the vitamin is still unknown. This subject will be fully discussed later under the headings "folate absorption", "folate content of foods" and "bioavailability of food folates".

Biosynthesis of pteroylpolyglutamates. Our understanding of the biosynthetic process of pteroylpolyglutamates was long hindered by the unavailability of sophisticated analytical techniques. Early methodologies such as gel filtration and ion-exchange chromatography used to study the synthesis of pteroylpolyglutamates have recently been replaced by newly developed analytical techniques with higher degrees of precision and reliability.

In one such method developed by Shane (1982), the 9,10-carbon-nitrogen bond of folates was cleaved by acidic reductive or oxidative conditions and the resulting p-aminobenzoylpolyglutamates were separated by HPLC. The application of this method to the study of pteroylpolyglutamates synthesis in microorganisms has shown that octa- and nonaglutamates constitute the predominant forms of folates in Lactobacillus casei, while Streptococcus faecalis contained mostly tetraglutamates. The chain length of intracellular pteroylpolyglutamates in different animals has been found to be inversely related to the extracellular folate concentration (Brody et al., 1979)

and the equilibration of exogenous folate with endogenous pools has been proven to be a very slow process (Eto and Krumdieck, 1982). Those findings implied that the pattern of intracellular pteroylpolyglutamates in their steady-state would be different from the pattern just following the administration of radiolabeled folates. The fact that this is a widely used technique in the assessment of the vitamin's profile in humans raises serious questions about its validity.

Studies on the synthesis of polyglutamates have focused on the enzyme pteroylpolyglutamate synthetase (PPS) that catalyses the reaction:

$$H_4\text{-PteGlu}_n + \text{ATP} + \text{L-Glu} \text{ ----> } H_4\text{-PteGlu}_{n+1} + \text{ADP} + P_i$$

PPS was found to be 50 to 100 times more active in procaryotes than in eucaryotes (McGuire and Bertino, 1981), a fact that is still unexplainable since the pteroyl-glutamate pool of both classes is almost similar. With respect to its distribution, synthetase activity was detectable in most tissues except the kidney and the heart. The highest activity was reported in the liver and the cytosol was determined to be the major subcellular locus of mammalian liver synthetase.

Hydrolysis of pteroylpolyglutamates. Since folates appear in mesenteric circulation mainly as folyl monoglutamates, a hydrolysis step must take place within or

in relation to the intestinal cells. It is now clear that the hydrolysis of pteroylpolyglutamates is catalyzed by the group of enzymes called folate conjugases or more specifically γ-glutamyl hydrolases (EC 3.4.22.12). One of the early methods to assay for the activity of conjugases was to determine the product chain length by differential growth of Lactobacillus casei, which utilizes tri-, di- and monoglutamates, and Streptococcus faecalis that grows on mono- or diglutamates only. However, this method was found to lack absolute specificity (Tamura et al., 1972) and was largely replaced by a fast, simple assay using a radiolabeled terminal glutamate that can be quantitated by liquid scintillation techniques (Krumdieck and Baugh, 1970). Recently, Priest et al. (1982) developed a very sensitive assay of -glutamyl hydrolase activity capable of detecting subpicomole amounts of hydrolysis products. These products were complexed with thymidylate synthase and tritiated fluorodeoxyuridylate and separated according to their charge by electrophoresis. Assessment of the chain length was then determined by densitometry of the fluorogram of the electrophoretic pattern. The advances made in the assaying procedures of conjugase activity have permitted an almost complete characterization of these enzymes obtained from different sources. γ-glutamyl hydrolase from E. coli had a pH optimum of 8.4 (Kosloff and Lute, 1981), while that of cabbage showed two different pH

optima, 5.0 and 8.0 . At a pH of 5.0 , the enzymatic hydrolysis of the polyglutamates was complete and resulted exclusively in monoglutamates, while at a pH of 8.0 the endproducts of the reaction were triglutamates. This raised the question of whether there were one enzyme with two different activities or two different enzymes (Tamura et al., 1972). The studies on animal conjugases have been limited almost exclusively to mammals, where hydrolase activity has been detected in the spleen, brain, liver, kidney and several tissues of reproduction, particularly the uterus and the placenta (Krumdieck et al., 1976). A study performed by Priest et al. (1982) found the highest hydrolase activities in kidney and muscle tissues, and the lowest in the liver and brain. The fact that liver hydrolase resulted in monoglutamate endproducts, while all other hydrolases showed intermediate glutamates, suggested the possibility of its action as an endopeptidase with specificity to the first γ-glutamyl bond. Hydrolase activity was also detected in human blood, lymph and plasma. The occurrence and activity of γ-glutamyl hydrolase in the digestive system will be separately discussed in the next section dealing with folate absorption.

With respect to subcellular distribution, the highest hydrolase activity appears to be associated with the lysosomal fraction of most tissues. This is particularly

true of liver which has been proposed as the probable source of the γ-glutamyl hydrolases found in physiological fluids (Horne et al., 1981). However, no definite answer can be given yet as to the origin of plasma hydrolase nor, to the regulation of its activity. The latter is thought to be effected mainly by the length of the polyglutamate chain through its influence on enzyme kinetics, or by such other factors as estrogen level and DNA synthesis (Krumdieck et al., 1975).

Generally speaking, the γ-glutamyl hydrolases obtained from different sources appear to have similar enzymological properties: longer polyglutamates are usually the preferred substrate and there is an absolute specificity for the peptide bonds involving the γ-carboxyl group of the glutamate, irrespective of the substituent on the α-amino group. Moreover, there is strong evidence that the hydrolase is non-specific with respect to the pteridine moiety (Silink et al., 1975). Further characterization of the enzyme has been very controversial. Some researchers have observed intermediate polyglutamates in the reaction mixture, and concluded that the enzyme is an exopeptidase (carboxypeptidase), while others have reported that it is an endopeptidase (hydrolase) attacking the polyglutamate chain in a random fashion. Also, controversies have surrounded the subject of potential inhibitors and activators of the enzyme. The possible inhibition of

intestinal hydrolase by drugs, such as anticonvulsants and oral contraceptives, has caused much concern due to its possible role in folate deficiency.

Folate Absorption

Naturally occurring folates exist mostly in the polyglutamate form while the circulating form of the vitamin in biological fluids is monopteroylglutamic acid and its derivatives. The mechanism of folate absorption is, therefore, coupled with a deconjugation process that takes place in the digestive system and uses the enzyme -glutamyl hydrolase. Although detected in human saliva, stomach mucosa (Bernstein et al., 1970) and gastric juice, very little hydrolase activity has been detected in intestinal juice, even after stimulation of pancreatic and bile secretions. However, the presence of conjugase activity in mammalian intestinal mucosa suggests an intracellular hydrolysis with very little brush border activity (Halsted et al., 1976). In their study of folate absorption in dogs, Baugh et al. (1975) confirmed such an hypothesis and considered the hydrolysis of pteroyl-polyglutamates part of their absorptive process. The sequence of this process appears to be mucosal uptake, progressive hydrolysis to pteroylmonoglutamates and final exit from the mucosal cell. The same researchers also used the method of jejunal perfusion to study folate digestion in six human patients with tropical sprue. Since the

products of pteroylpolyglatamate hydrolysis were the same before and after treatment, and since the disease was known to affect the brush border surface, it was concluded that this surface was not the site of hydrolysis. Futhermore, folate malabsorption in tropical sprue is not the result of decreased conjugase activity.

In spite of numerous studies on the subject, the mechanism of folate absorption is still far from being resolved. Newly developed techniques capable of detecting lower enzyme activity are proving to be very helpful in this area. A study by Reisenauer et al. (1977) indicated that the human mucosa actually contains two hydrolases: a soluble intracellular lysosomal enzyme and another which is membrane bound and located in the brush border. The latter had not been detected earlier, due to its low activity. It is postulated to be the actual enzyme involved in absorption. Our current concepts of the biological processes of folate absorption and malabsorption are not very different from the ones voiced by Rosenberg (1977) about ten years ago. He stated that "the site at which the polyglutamyl folate is hydrolysed has not been determined, but after hydrolysis the product enters a common pathway of absorption with monoglutamyl folate and to some degree is converted to methyl folate. The rate of hydrolysis substantially exceeds the overall rate of transport. Thus, only a severe effect on hydrolysis would result in a

slowing of transport, whereas any direct effect on the transport process would cause malabsorption of dietary folate".

In recent years, studies on folate therapy revealed much needed information on the absorptive process of this vitamin. Botez and Bachevalier (1981a) investigated the absorption of folic acid under various clinical conditions. A significant difference was detected between normal subjects and patients with atrophy of the jejunal mucosa. No such difference was reported between controls and diabetic patients. Poncz et al. (1981) looked into the problem of congenital folate malabsorption. Their findings suggest that the transport of folinic acid in the body is more efficient than that of folic acid. Therefore, the former should be the derivative of choice in treating congenital folate malabsorption. A recent study by Kesavan and Noronha (1983) found that the intestinal mucosal conjugase did not play a significant role in the physiological absorption of dietary folates. The study was done on aged rats, concluding that folate malabsorption was related to low levels of pancreatic folyl conjugase.

Role of Folates in Metabolism

The major role of the coenzyme forms of folacin is in the transfer of one-carbon units required for the synthesis of DNA, RNA, methionine and serine. The physiological effects are reflected in the proper formation and

maturation of red and white blood cells in the bone marrow (Krause and Mahan, 1979, p. 173). Specifically, folacin acts as a single carbon carrier in the formation of heme and plays an important role in the synthesis of the purines guanine and adenine, necessary components of the nucleoproteins. In this respect, purine biosynthesis can be indirectly blocked by antibacterial agents of the sulfonamide class, which are structural analogues of p-aminobenzoic acid, and competitively inhibit the formation of folic acid (Lehninger, 1978, p. 732).

The participation of folates in the synthesis of the pyrimidine thymine, which is utilized in the formation of DNA, is highly important also. Thus, the conversion of deoxyuridilic acid (dUMP) to deoxythymidylic acid (dTMP) is catalyzed by thymidylatesynthetase which requires 5,10-methylenetetrahydrofolate as the coenzyme:

$$\text{dUMP} \xrightarrow{\text{Thymidylate synthetase}} \text{dTMP}$$

$$5,10\text{-}CH_2\text{-THF} \longrightarrow \text{DHF}$$

Since the activity of thymidylate synthetase is sensitive to depressed levels of tetrahydrofolate, the latter becomes a limiting factor in the synthesis of DNA. Tetrahydrofolate is regenerated from dihydrofolate according to the following reaction:

$$\text{NADPH} + H^+ + \text{DHF} \xrightarrow{\text{Reductase}} \text{NADP}^+ + \text{THF}$$

Therefore, agents which bear structural resemblance to DHF are competitive inhibitors of the reaction and greatly retard DNA synthesis (Lehninger, 1978, p. 739). This property of anti-folate drugs such as aminopterin and amethopterin is used in cancer therapy. The greatest success has been reported in the slowdown of the growth of leukemic cells.

Other metabolic roles of folates include their participation in the interconversion of serine and glycine, the methylation of ethanolamine to choline, the methylation of nicotinamide, the conversion of histamine to glutamic acid, the oxidation of glycine, the oxidation of phenylalanine to tyrosine and the methylation of homocysteine to methionine. The latter reaction requires the presence of Vitamin B_{12} (cyanocobalamine) as a cofactor which has produced many theories as to the regulation of folate metabolism by cyanocobalamine. Recent theories have related alterations in the transport of folic acid through membranes to a cobalamine deficiency. However, the "methyl trap" hypothesis proposed earlier by Noronha and Silverman (1962) and by Herbert and Zalusky (1962) still seems to offer the best explanation of the interrelationship of folic acid, Vitamin B_{12} and methionine. The theory holds that the reduction of 5,10-CH_2-THF to 5-CH_3-THF is almost irreversible. Therefore, the demethylation of methyl folates is only possible by their reaction with

homocysteine through the Vitamin B_{12} dependent transferase to yield methionine. The "methyl trap" hypothesis was reviewed by Stokstad (1977), who summarized the interdependency of the above metabolites in the schematic diagram shown in Figure 3. It is readily apparent from the diagram that B_{12} deficiency would result in the accumulation of 5-CH3-THF and an increased excretion of formiminoglutamic acid (FIGLU) as well as imidazolecarboxamide. The situation could be restored to normal by supplementation of dietary methionine as proven by numerous research papers on the subject .

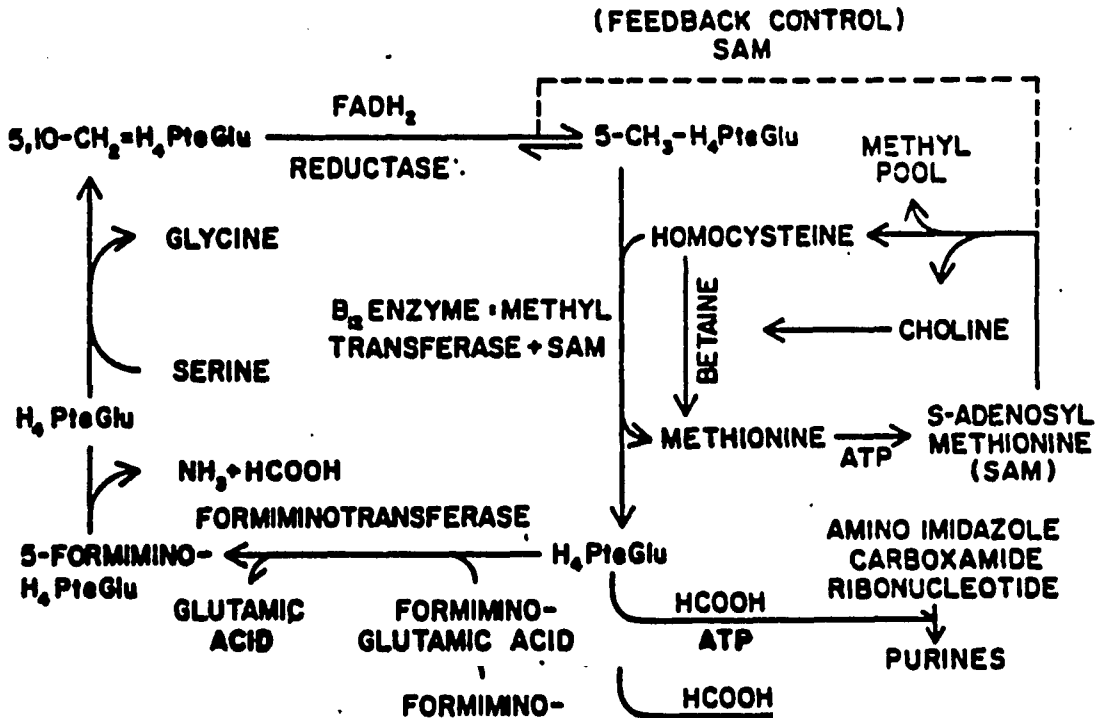


Fig. 3-- Interrelationships between folic acid, Vitamin B_{12} and methionine.

Interconversion of Folate Derivatives

Since various folates might differ in their biological activity, the interconversion of folate derivatives gains importance with respect to its effects on metabolism and on the availability of dietary folates. Mackenzie (1984) suggested that serine and glycine are the common sources of one-carbon units in most cells, and therefore entry to the active C_1 pool of intermediates is via 5,10-CH2-THF. The latter can be used directly for the synthesis of thymidylate or further metabolized by complex enzyme systems.. In that case, it can either be reduced to 5-CH3-THF for the biosynthesis of methionine or oxidized to 10-HCO-THF for purine synthesis. Also, 10-HCO-THF could be the product of folate utilization as a one carbon source. It would then require reduction for use in the synthesis of serine, methionine and thymidylate. A complete picture of the interconversion of substituted tetrahydrofolates was given by Witter and Wagner (1981) (Fig. 4). Recently, Foo et al. (1982) reported that more than 95% of the intracellular folate derivatives are polyglutamates of chain length varying from 2 to 10 glutamic acid residues. Also, it was determined that when fibroblasts were cultured with folic acid, the major derivatives formed were hexaglutamates. By contrast, heptaglutamates resulted when the fibroblasts were cultured in the presence of methyl-THF. In a study originally intended to evaluate the

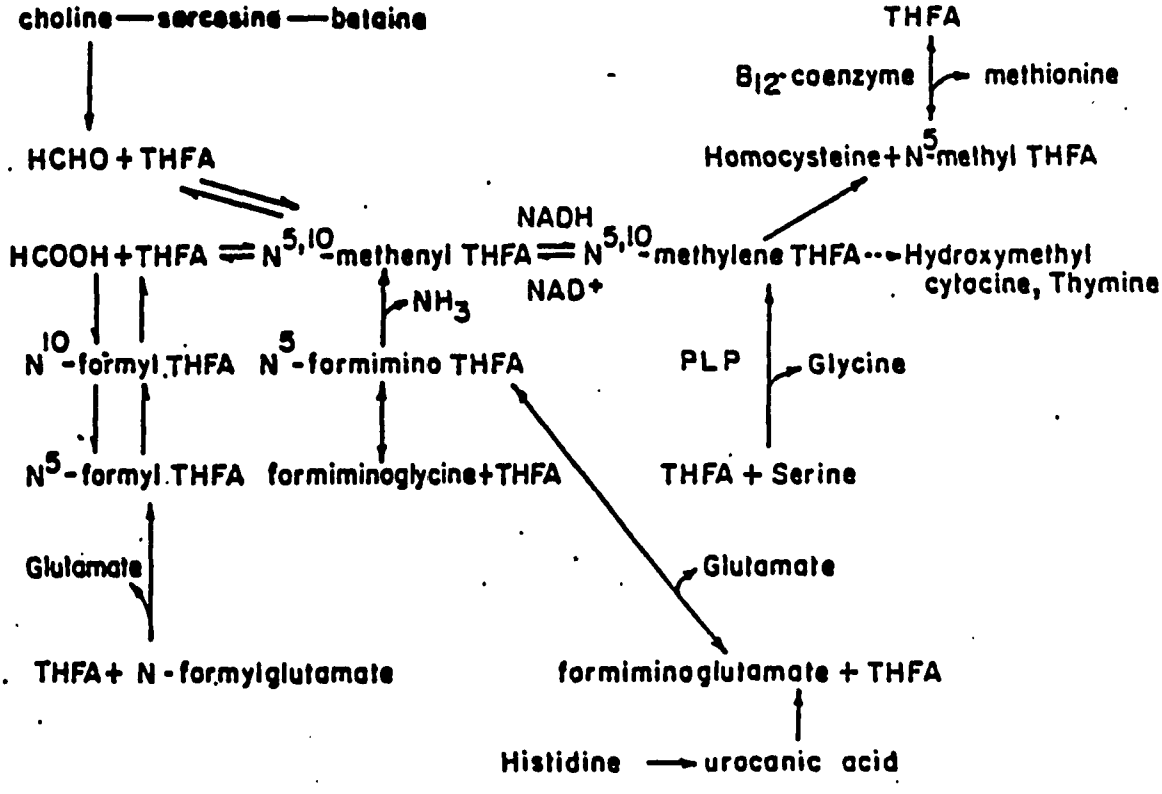


Fig. 4--Interconversion of folate derivatives

protective effect of ascorbic acid on folates, Wilson and Horne (1983) reported intensive interconversions of folates upon heating for 10 mins. at 100°C in 2% sodium ascorbate.

Products of Folate Catabolism

The identification of folate catabolites in vertebrates has been complicated by the controversy over the exact rate of folate catabolism. Labeled material excreted in the urine over the first two days following administration of radioactive folates is now believed to consist of active metabolites of folic acid degraded during their passage through kidneys or intestines (Scott, 1984). These compounds would not be true catabolites. Moreover, there is increased evidence suggesting that the incorporation of dietary folate into human cellular pools might well take more than four days. Most of the studies dealing with the catabolism of the vitamin have attempted to identify and quantitate the catabolites excreted in the urine after ingestion of an oral dose of radiolabeled folates. In this respect, an extensive study by Barford and Blair (1975) found the following radioactive material in the collected urine of rats administered labeled doses of folates : 10-CHO-Folate, unaltered folate, 5-CH_3-THF and a degradation product of the latter believed to be 4a-OH-5-CH_3-THF. The absence of p-aminobenzoylglutamic acid, usually produced through cleavage of the C9-N10 bond of folate metabolites, was later demonstrated to be due to a further acetylation to p-aminobenzoylglutamate. Other urinary catabolites of folates proposed by different researchers include 10-HCO-Folate, 10-HCO-H_2-Folate, 5-CH_3-

H_2-Folate and 4a-OH-5-CH_3-H_4-Folate. The pterin products of folate catabolism deserve special attention since numerous studies have suggested that tumor cells produce abnormal quantities of specific pterins. In this respect, the pterin metabolites of healthy cells are reported to consist mainly of biopterin and D-erythropterin, with trace amounts of threoneopterin, isoxanthopterin, sepiapterin and pterin (Fukushima and Shiota, 1974). On the other hand, high levels of pterin-6-carboxaldehyde, 6-CH_2OH-pterin, xanthopterin, neopterin and biopterin have been reported in malignant cells (Scott, 1984). Since the compounds just mentioned are not usual products of folate catabolism, they probably arise from altered folate mechanisms rather than increased catabolic processes in cancerous cells. Folate deficiency in alcoholism, pregnancy and anticonvulsive therapy has been attributed to alterations in folate metabolism. This aspect of folate catabolism will be the subject of detailed discussion in the following section.

Folate Deficiency

An adequate supply of folacin is usually easy to obtain from a well balanced diet. However, folate deficiency is not uncommon in situations of increased needs, to be discussed later, as was documented in the Ten-State Nutrition Survey of 1968-1970 (1972). Folic acid deficiency was reported by Rosenberg and Dyer (1979) who discussed its prevalence and possible causes in the United

States. Inadequate intakes of the vitamin are believed to result in poor growth, megaloblastic anemias or other blood disorders, glossitis and gastrointestinal disturbances. Other symptoms that also respond to folic acid therapy include diarrhea, irritability, forgetfulness, weight loss and palpitations (Goodhart and Shils, 1973). Recently, folate deficiency has been implicated in the susceptibility of infants to malarial infection (Brabin, 1982) and with a degenerative effect on the reproductive organs of female Rhesus monkeys (Mohanty and Kshitish, 1982). Moreover, experimental folate deficiency in rats resulted in fatty liver and decreased biosynthesis of hepatic phospholipids (Akesson et al., 1982). In guinea pigs, lower concentrations of ascorbic acid in the adrenal glands and the liver were associated with folic acid deficiency (Lewis et al., 1982). However, contrary to previous observations in humans, there was no change in erythrocyte filterability or in membrane proteins (McGown et al., 1982). Recently, extreme concern has been expressed regarding the presence of folate sensitive fragile sites on the human chromosomes. Those were defined by Djalali et al. (1985) as "specific chromosomal loci which were manifested as gaps and breaks in a number of cells under conditions of <u>in vitro</u> folate deficiency". In this respect, Reidy et al. (1983) reported that chromosome breakage was significantly lower in a culture medium containing folic acid than in a medium that

did not. Future research should be directed towards studying the implication of such a finding on genetic expression in humans.

Defining folate deficiency is still a problem since there is no general agreement as to how to assess folate status. The World Health Organization (WHO) guidelines (1972) for the evaluation of nutritional data considered that with serum folates in the range of 6 to 20 ng/ml there is less than a 3% probability of developing a deficiency. Also, the guidelines suggest that RBC levels are a more accurate reflection of folate status than serum folates. A reliable assessment of folate nutriture requires measurements of serum and whole blood folate levels and an RBC folate calculation, based on the hematocrit value. A similar attitude was recently expressed by Stanley et al. (1984), who suggested the use of more than one index of folate nutriture; since the true folate status is reflected neither by serum folate nor by 24 hour dietary record folate nor by longer term indexes such as RBC and food frequency folates.

Laurence et al. (1982) reported a strong association between quality of diet and RBC folate. The association was much smaller with serum folate levels which actually would be a measure of the folates in transit. Kraus and Mahan (1979) indicated that folate deficiency is manifested by serum levels of less than 3 milligrams per liter or RBC

levels of less than 100 milligrams per liter. Another possible index of folate defiency is an increase in the urinary excretion of formimino glutamic acid (FIGLU). Kraus and Mahan (1979, p. 173) also considered proper diagnosis a very important step, especially in those cases where a B_{12} deficiency is suspected. The administration of folate in such a case would have a masking effect on the symptoms of Vitamin B_{12} deficiency and progressive nerve disease could go unnoticed. For this reason, folic acid is still excluded from many of the commercial vitamin formulas and some physicians prefer to rely on a combined folate-B_{12} therapy for the treatment of megaloblastic anemia.

Folate deficiency should be of prime concern during pregnancy. This is especially true of low income pregnant women, as proven in studies performed in New York City (Herbert et al., 1975), Los Angeles (Jacob et al., 1976) and North Central Florida (Bailey et al., 1980). Also, similar findings have been reported in black and Spanish-American adolescents from urban low-income households (Bailey et al., 1982).

Rosenberg et al. (1982), who investigated folate nutrition in the elderly, reported a generally adequate intake of the vitamin in this group. Malabsorption was not found to be a common problem. Concern about folate nutriture in the elderly has been primarily directed at hospitalized patients and alcoholics.

Increased folate requirements have been documented during lactation, infancy, cirrhosis, protein-calorie malnutrition, topical sprue, scurvy, malignancies and following intestinal or jejunal resection. Recently, folate deficiency has been linked to the ingestion of various therapeutic drugs. Girdwood (1977), who reviewed the subject of drug-induced folate deficiency, classified those drugs under five main headings: those that interfere with dihydrofolate mechanism, such as aminopterin and methotrexate; drugs which interfere with DNA synthesis, such as 5-fluorocaril; antipiletic drugs, such as phenytoin; drugs which interfere with Vitamin B_{12} absorption, such as Metformin; and finally, drugs of doubtful action on folates, which include mainly oral contraceptives and ethyl alcohol.

The exact mechanism by which some of these drugs precipitate folate deficiency is still unknown. However, it is believed to be associated with either increased folate catabolism or impaired folate absorption. In any case, if folate stores are to be replenished, daily doses of 1 milligram of folic acid are given orally for two to three weeks. Hematologic and symptomatic improvements should then be clearly evident.

Folate therapy has been successfully used in the treatment of anemia during pregnancy in Liberia (Jackson, 1982) and in cases of cervical dysplasia in users of oral

contraceptives (Butterworth et al., 1982). Moreover, Botez and Bachevalier (1981b) used folic acid supplements to treat 23 patients with folate-responsive neurological disorders. They reported that contrary to previous statements, there is no absolute brain-barrier for folate.

The use of folate supplements at therapeutic levels raises the question of folate toxicity. While more research is needed in this area, there has been some reports of psychiatric changes associated with an excess of folic acid (Prakash and Petrie, 1982). Also, it has been reported that zinc excretion was reduced by 50% due to folic acid supplementation. No changes were noted, however, in iron or copper excretion patterns (Milne et al., 1984).

Methods of Analysis

Several methods have been developed for the analysis of folates. However, most of those methods are only suited for the evaluation of folate activity in pure or pharmaceutical preparations. When biological tissues are considered or a qualitative profile of folate derivatives is sought, complicated fluorometric or chromatographic techniques have to be used.

Colorimetry. In the colorimetric method, standards of folic acid are compared spectrophotometrically to solutions of the sample to be analyzed. The folate concentration should be of the order of 0.1 mg/ml and the sample is

usually a pure pharmaceutical preparation.

Fluorometry. Fluorometric techniques are more sensitive and more suitable for biological samples than colorimetric ones. However, care should be taken to clean up the sample to remove interfering fluorescent compounds such as xanthopterin and tyrosine.

Chemical. A method based on the quantitation of the cleavage products of folates after acidic reduction, mainly p-aminobenzoylglutamic acid and methylpteridine was reported by Blakley (1969). The method is only suitable for non-biological material with concentrations in the range of 5 to 20 micrograms.

Enzymatic. Originally intended to identify folate derivatives, this method relies on the use of enzymes that catalyze the reactions of folate interconversions. Basically, it consists of comparing the reaction rate of the sample material to that of a known substrate (Blakley, 1969). This method has largely been replaced by more efficient chromatographic techniques.

Animal assays. The most common animal assays of folate use chickens, monkeys, guinea pigs, rats and dogs. Although not very sensitive, animal assays have proven very effective in determining the activity of folate derivatives in a given organism. They have been valuable in assessing the availability of folates from specific dietary sources. Recently, Keagy and Oace (1982) developed a new folacin

bioassay in rats. They reported that the concentration of folacin in the liver is suitable for use as the response variable in a folate bioassay.

Radiometric assays. Competitive binding radiometric assays are widely applied in the analysis of blood folacin. Reported by Rothenberg et al. (1972) as an implication of the discovery of folate-binding proteins, such assays were improved to measure folates in biological tissues (Tigner and Roe, 1979) and frozen foods (Graham et al., 1980). Recently, Chen et al. (1983) used a combined radiometric microbiological method to measure the folacin contents of foods. Moreover, Gregory et al. (1982) compared radiometric assays to microbiological and HPLC techniques. Their results suggested that reverse phase HPLC provides the best methodology of folate analysis.

Microbiological assays. Despite current advances in chromatographic and radiometric techniques, microbiological assays are still widely used in assessing the folate activity of foods and biological tissues. These assays have been used since the identification of the Lactobacillus casei factor and the Streptococcus lactis R factor, both proven to be folate derivatives. Originally, the three organisms most commonly used in the assay were Lactobacillus casei ATCC 7469, Streptococcus faecalis ATCC 8043 and Pediococcus cerevisiae ATCC 8081. Later, it was found that L. casei should be the organism of choice, since

its response to folates is very similar to that of man (Keagy et al., 1975). By contrast, S. faecalis does not respond to the man-utilizable methylfolate, but can metabolize the non-utilizable pteroates. Recently, Wilson and Horne (1982) used glycerol-cryoprotected L. casei for the microbiological assay of folic acid. The organism was grown in a medium which contained 0.3 µg/l folate and was diluted with an equal volume of glycerol (800 ml/l) before being frozen at -20°C. This method was reported to be an improved microbiological assay.

Recently, the microbiological assays of folates have come under increased criticism (Phillips et al., 1982). They are reported to yield different results when different sources of deconjugase enzymes are used (Phillips and Wright, 1983). Moreover, 5-CH3-THF was shown to give a lower response than pteroylglutamic acid, the vitamer used as the standard in the assay (Landrigan, 1982). With these facts in mind, the proliferation of HPLC techniques in future work on folates is highly possible.

Chromatography. Column, paper and thin-layer chromatography have all been used to separate and quantitate folic acid derivatives. However, the use of these traditional chromatographic techniques, often tedious and time consuming, have been offset in recent research which has focused on the use of high performance liquid chromatography (HPLC). Due to their ability to achieve

fractionation of complex mixtures of folates in a relatively short time, HPLC methods have been developed for folate analysis.

Reed and Archer (1976) used HPLC to separate the reduced and N5 or N10 substituted folates. The column consisted of a pellicular, weak anion-exchange material (Al-Pellinox-Wax) with 0.025 M sodium dihydrogen phosphate, pH 4.8, or 0.006 M sodium dihydrogen phosphate, pH 4.0 as the mobile phase. The flow rate was maintained at 1.15 ml/min at pressures of about 1000 psi. UV detection at 254 nm was used. Standard curves, obtained by plotting peak areas versus the amount of folate derivative injected, were linear over a range of 1 to 20 ug. However, the study was limited to commercial standards of folates and did not extend to biological material and food substances. In another study, Clifford and Clifford (1977) established optimum conditions for the separation of a mixture of THF, 5-CH_3-THF, DHF and folic acid on a pellicular strong anion exchange resin using a potassium chloride gradient in phosphate buffer at a pH of 7.5 and a temperature of 40°C. The flow rate was maintained at 0.25 ml/min. Separations using this method were dependent on the substitution of the pteridine ring but independent of the glutamic acid chain length. The results were found to be linear and reproducible. The method was later applied to determine the folate profiles of lemon, grape and apple juices,

almonds, wheat germ, and raw peanuts. Folate extraction of the above samples was accomplished by homogenizing with 1 to 5 volumes of 0.01M potassium phosphate buffer, pH 7.5 containing 0.2M mercaptoethanol, followed by immersing in a boiling water bath for 3 minutes before being quickly chilled on ice. The study produced a rapid reliable HPLC procedure for the analysis of food folates, but was limited to only four derivatives of folic acid.

A different approach, reported to be particularly suited to biological systems, was used by Reingold et al. (1980) to separate folate derivatives by an in-situ paired-ion HPLC process. To overcome the problem of endogenous sample components co-chromatographing with the different folates, the authors equilibrated the u-Bondapak phenyl column with PIC A-phosphate buffer prior to injection. Ion-pairs of the various folates that were formed in-situ were retained on the column until interfering substances were eluted with the initial perchlorate-phosphate buffer mobile phase. This method was found to be more time effective than the traditional clean-up procedures of biological material. It was successfully used to separate standards of folic acid, 7,8-DHF, 5,6,7,8-THF, 5-CH_3-THF and p-aminobenzoylglutamic acid. Application of this method for the quantitation of the folacin content of various foods is reportedly under way.

Also, ion-pair chromatography was also used by Allen and Newman (1980) for the qualitative and quantitative separation of folic acid, DHF, 5-HCO-THF, 5-CH_3-THF and p-aminobenzoyl-L-glutamic acid. A Spectra-Physics, ODS 5 um was employed and was eluted with a degassed solution of methanol and water each containing 0.005M tetrabutyl-ammonium phosphate. The flow rate was 1.0 ml/min and separation was achieved with a gradient. The standard curves were obtained by plotting peak height versus the amount of folate injected. These conditions yielded results which were reproducibly linear over a 10-fold concentration range.

A more recent study by Shane (1982) focused on the identification of the polyglutamate chain lengths of labeled and unlabeled folates in biological extracts. Folates were cleaved to p-aminobenzoylpolyglutamates, converted to azo dyes and purified by chromatography on Bio Gel P2 polyacrylamide before being separated by HPLC (Partisil 10 SAX anionic exchanger). Folates were detected and quantified by their absorbance at 280 nm. By this procedure, rat liver was found to contain a mixture of pteroylmono- to heptaglutamates with the pentaglutamates predominating. In other studies, Gregory et al. (1984) described the fluorometric determination of folacin in biological materials using HPLC. Tani and Iwai (1983) separated 15 folate derivatives and oxidation products by

HPLC on a Cosmosil 5-Ph column. They used their procedure to prove that folic acid is converted to 5-CH_3-THF in the small intestine of the rat during absorption.

Recently, a reverse phase HPLC method was developed by Day and Gregory (1981) for the separation and quantitation of the major folate derivatives in foods and other biological materials. Sample extract purification was improved by a preparative chromatographic method using Bio-Beads SM-2 hydrophobic resins. The coupled analytical columns used consisted of an Altex Ultrasphere IP followed by a u-Bondapak phenyl and were protected by a Whatman silica precolumn. Pt-6-COOH and the monoglutamate forms of THF; 7,8-DHF; 5-CH_3-THF; 5- and/or 10-HCO- were isocratically separated by an acidic phosphate-acetonitrile mobile phase. Detection was performed by UV absorbance at 280 nm or by a postcolumn fluorogenic oxidative derivatization method which enhanced specificity for THF, DHF and folic acid. The folate profiles of beef liver, fortified breakfast cereal and a liquid infant formula were obtained by this procedure.

Dietary Folates

Distribution and availability. Although studies on the folacin content of foods date back to the early 1950's, a surge of interest in the quantitation of dietary folates did not occur until 1970. This was probably due to the

inclusion of folic acid in the 1968 RDA. The task of quantitating folates has not been an easy one. Also, the accuracy of the data on food folates is still debated (Phillips et al., 1982). The folacin content of foods has been determined mostly by microbiological assays. Such assays are subject to numerous interfering factors which could compromise the data. One such factor is the variability in the response of different assaying organisms to the individual folates. Thus, when Dong and Oace (1973) investigated the folacin content of bovine milk, they reported a value of 7.7 µg/100 ml when the assaying organism was L. casei. When S. faecalis or P. cerevisiae were used, the folacin content of the same milk sample was reported as 0.028 and 0.30 ug respectively. Another factor that affects the results of the microbial assays is the use of ascorbate as a protective agent during the procedure. Many researchers now feel that all food tables prepared prior to the use of ascorbate in the assaying medium are underestimates of the actual values. Therefore, such food tables should be reviewed (Hoppner et al., 1977).

Another consideration is the large discrepancy normally found between the total and the free folate values. The total folate content of a food refers to the value obtained by the conventional microbiological assay which had been preceded by a conjugase treatment of the food extract. This treatment is directed towards the

hydrolysis of the pteroylpolyglutamates at the -glutamyl linkage. The result of this deconjugation step is the release of mono- and diglutamates of folic acid which would be readily utilizable by the assaying organism. By contrast, to obtain a value for free folates, the microbiological assay would be run on the food extract with no conjugase treatment. Then, pteroylpolyglutamates are mostly unaccounted for. The discrepancy between total and free folate values can be seen in a study by Santini and Corcino (1974) on the folic acid content of Puerto Rican diets. In their results, the authors reported a range of 37-130 ug for free folacin and one of 1558-3156 ug for the total folacin content of a high cost diet. Herbert (1961) has suggested that free folate values from the L. casei assay constitute the best approximation of the availability of this vitamin in foods. However, conjugase activity has been reported in both animal and vegetable tissues, which has two implications. Firstly, the free folate content of raw products might be overestimated due to conjugase activity. This was suggested by Malin (1976) who also reported that losses in free folates upon cooking might actually reflect higher concentrations of coenzymes in raw products rather than thermosusceptibility of the vitamin. Secondly, there is the possibility that pteroylpolyglutamate hydrolysis occurs in the GI tract. The extent of such hydrolysis has not been determined as

yet, and is not accounted for in free folate determinations. However, conjugase activity has been detected in human saliva, gastric juice (Jagerstad et al., 1976) and intestinal mucosa (Halsted et al., 1976). Therefore, free folate values might actually be underestimates of the actual activity of dietary folates.

Other factors that may interfere with a correct appraisal of folates by the traditional microbiological assay include exposure to light, pH of the extracting buffer, the response of L. casei to different folate monoglutamates (Phillips and Wright, 1982) and the source of the conjugase used to hydrolyze the polyglutamates. In this respect, a recent study by Kirsch and Chen (1984) was performed to compare the conjugase treatment procedures of food folacin. The results of this study showed no significant effect of the source of conjugase on the assay. However, other variables affected its outcome. These included the nature and pH of the extracting buffer, total incubation time and method of sample filtration.

Factors that interfere with the standardization of folate assays are only one aspect of the problem. The assessment of folate availability in foods is hindered by the natural occurrence of various forms of folates and conjugases in biological tissues (Gregory et al., 1984b). Differences in the activity and stability of those forms in the acidic environment of the stomach are not fully

documented as yet. Consideration of the presence of conjugase inhibitors in foods or in the intestinal tract has been neglected, also. Therefore, the reliance on folate excretion tests to measure the availability of this vitamin in foods is justifiable in spite of the time and effort it usually involves. Using such a test, Tamura and Stokstad (1973) reported low availability of folates in Romaine lettuce (25%), orange juice (31%) and egg yolk (39%). Higher availability was reported for the folates of bananas (82%) and frozen lima beans (96%). Other studies have indicated green leafy vegetables, liver, peanuts and wheat germ as good sources of the vitamin.

The availability of folates have been questioned in high fiber diets as well. But recent research indicates that dietary fiber is not likely to effect the utilization of folic acid from foods (Ristow et al., 1982a; Keagy and Oace, 1984). Likewise, the presence of folate-binding protein in bovine milk is currently being investigated as to its effect on folate availability (Tani and Iwai, 1984).

A detailed appraisal of folacin intake, by the L. casei method, was reported by Hoppner et al. (1977) based on the examination of a composite Canadian diet. The findings relate to composites which contained 64% water and provided 88 g of protein and 2780 Kcal/day (Table 3).

Table 3. The folate content of various food groups

Food group	Intake g/person/day	Folacin, ug Free	Total
Dairy products	493.4	19.2	33.6
Meat, poultry, fish	194.0	32.0	59.2
Cereal products	260.0	20.1	36.2
Potatoes	178.7	5.7	21.3
Leafy vegetables	38.8	4.9	5.6
Root vegetables	38.4	0.9	1.5
Legumes	31.7	7.0	14.8
Garden fruits	79.6	9.4	10.4
Fruits	160.4	1.9	3.4
Oils and fats	26.6	0.9	3.5
Sugars and adjuncts	158.3	1.1	4.1
Total	1660.3	103.1	193.6

A similar study on the folic acid content in the British household supply (Spring et al., 1979) reported values of 88 ug and 165 ug for the free and total folic acid, respectively. It is interesting to note that these levels are much below the recommended daily intakes in Canada and the U.S. Also, folate deficiency is not a common nutritional problem in the U.K. This prompted Bates et al. (1982) to address the issue of the discrepancy between normal folate intakes and the folate RDA. The folate intake of adolescents was investigated by Bailey et al. (1984). They reported that the poor folacin status of rural black and Hispanic adolescents is a reflection of the infrequent consumption of vegetables and fruits. The

folate status of adult males living in a metabolic unit, and its possible relation to iron nutriture, was discussed by Milne et al. (1983). These researchers found that adjustments in body folates reflected dietary intakes. Moreover, an intake of about 200 ug/day of folates appeared to be sufficient enough to maintain body stores of the vitamin. In recent years numerous studies have appeared on the folate content of foods in various parts of the world. Huq et al. (1983) reported the folacin content of various Nigerian foods, while Poh Tan et al. (1984) presented the folic acid content of the diet in various types of the British household. Chen et al. (1983) indicated that 5 cups of tea per day would provide about 25% of the RDA of folic acid. Cooperman et al. (1982) reported the values of 16.3 ng/ml and 33.4 ng/ml of folic acid in transitional and mature human milk, respectively.

Liver Folates

Early studies on liver folates were directed primarily at the separation and identification of the different folic acid compounds. Such studies, performed mainly on rats, revealed that the folates of liver consist mainly of pteroylpolyglutamates. Shin et al. (1972) reported that 85 to 90 percent of liver folates in the rat are reduced forms of pteroylpentaglutamates. The major derivatives identified were 10-HCO-H_4-$PteGlu_5$, 5-HCO-H_4-$PteGlu_5$, 5-CH_3-H_4-$PteGlu_5$ and H_4-$PteGlu_5$. The folates were extracted by

dropping slices of liver in a boiling 1.1% ascorbate solution (pH 6.0) and then separated by gel filtration on Sephadex G-15. Similar findings were reported by Day and Gregory (1981) who used HPLC techniques to identify and quantitate folic acid derivatives in beef liver. They indicated that THF (0.78 µg/g), 5-CH_3-THF (0.76 µg/g) and 5-HCO-THF and/or 10-HCO-THF (1.87 µg/g) were the major folates present. Contrary to rat liver, folates in beef liver were studied for their contribution to the dietary intake of the vitamin. Liver is a very rich source of folic acid. It is reported to contain 145 ug (Hoppner et al., 1977) to 292 µg (Day and Gregory, 1981) of highly available folate per 100 grams. Therefore, inclusion of liver in the diet could play a major role in helping to meet the daily requirement of the vitamin.

Effects of Processing and Storage on Folic Acid

Compared to other nutrients, very few studies have been carried out to evaluate the effects of processing and storage on folic acid. This is due, in part, to the complexity of folate assays and to the fact that the inclusion of folic acid in the RDA tables is relatively recent (1968). Early work by Burton et al. (1970) compared the vitamin composition of milks sterilized by different processing techniques. The destruction of folate was found to be closely associated with that of ascorbate, which provided a stabilizing effect on folic acid. Deoxygenation

of the milk reduced the losses of both vitamins. Moreover, the injection of steam for HTST sterilization caused only a 7% loss as compared to 39% for the sterilization of milk in bottles. Another study by Hellendoorn et al. (1971), on meat and vegetable stew, found negligible losses in folate activity due to canning and storage for 5 years. The extent of folic acid losses during the canning process of garbanzo beans was evaluated by Lin et al. (1975). There was a 30% loss in total folates after processing at 118°C for 30 min. Also, steam blanching was superior to water blanching with respect to folic acid retention. The folate content of bread has received special attention due to its suitability for fortification purposes. Keagy et al. (1975) reported an initial increase in the folic acid content of dough due to the activity of the yeast. However, at the end of the bread making process, 31% of the folates were lost. The fate of folates in microwave and conventional heating was investigated by Cooper et al. (1978). 5-HCO-THF was found to be very stable at 100°C in aqueous solution, while 5-CH_3-THF and THF were significantly less stable. Microwave heating resulted in a faster rate of degradation for 5-CH_3-THF than conventional oven heating. The effects of canning and storage on the folate content of cowpeas, okra and tomatoes were examined by Sotiriadis (1978). Canning caused a 46%, 45%, and 43% loss, respectively in their folate contents. Addition of 2

mg/ml of ascorbate to the canning liquor provided a highly significant protection to the folates in all three products during canning. Ascorbic acid at 1 mg/ml provided only partial folate protection.

In more recent studies, Ristow et al. (1982b) studied the effects of thermal processing on folacin bioavailability in liquid model food systems, liver and cabbage. Their findings indicate that folic acid is stable during thermal processing at 120°C for 20 min while 75% of 5-CH_3-THF was degraded under the same conditions. Both folates were biologically available after processing as proven by microbiological and chick bioassays. Also, they found that the folacin from cooked beef liver appeared to be fully available, whereas 60% of that from cooked cabbage was not biologically available. In another study, Gregory et al. (1984) examined the activity of the two folacin oxidation products 10-HCO-FA and 5-CH_3-5,6-DHF. Both compounds are likely to be produced during processing of folate-containing foods. They were both shown to possess a high activity for Lactobacillus casei. Chick bioassays revealed 100% activity for 10-HCO-FA and 80% activity for 5-CH_3-DHF with respect to folic acid. Finally, a recent study by Bank et al. (1985) indicated that term or preterm human milk would not provide the RDA of folacin for infants if stored in the freezer for 3 months.

A potential hazard has been uncovered by Wogan et al. (1975) who suggested the possibility of formation of N10-nitrosofolic acid in processed foods. This compound has been shown to be a weak carcinogen in mice and may represent a health hazard to humans.

Folate Requirements

Herbert (1977) reported that the minimum daily requirement (MDR) for folic acid in adults is about 50 µg. He defined this requirement as the "quantity of PGA that, when administered orally or parenterally daily, to patients with folate deficiency uncomplicated by other systemic disease and not ingesting more than 5 ug of other dietary folate, will produce a relatively rapid return toward hematologic normality, accompanied by reticulocytosis and rise of hematocrit levels toward normal". It should be noted that with such a dose, serum folate levels would return to normal only after a period of one month. Also, many other studies have reported the MDR of folic acid to be about 50 ug and have recommended a PGA dose of 100 ug in the therapy of adult males with folate deficiency. This dose is based on the assumption that those subjects do not have increased folate requirements. However, folate deficiency is likely to develop in persons with increased requirements for the vitamins.

The situations of increased need for folates have been reviewed by Lindenbaum (1977). His list of conditions

necessitating higher folate intakes include: pregnancy, lactation, infancy, hemolytic anemias, alcohol ingestion and therapy with dihydrofolate reductase inhibitors. Other suspected conditions that have been associated with decreased serum folates include malignancies, anticonvulsant drug therapy, iron deficiency anemia, ascorbic acid deficiency and protein-calorie malnutrition. Moreover, a disturbance of the folate balance has been reported in cases of dialysis, intestinal atrophy, hyperthyroidism and oral contraceptive therapy.

The effect of contraceptives on folates has been the subject of intensive research. In a study investigating the hematologic abnormalities in adolescents on oral contraceptive pills, Grace et al. (1982) found a significant reduction in the serum and whole blood folate of white subjects but not in black adolescents. A similar study by Hettiarachchy et al. (1983) who reported that the administration of Ovulen 50 resulted in a significant decrease in serum folate levels. The decrease was rapid during the first six months of therapy, but then slowed down. Likewise, the work of Shojania (1982) showed that both serum and erythrocyte folate levels are lower in females using oral contraceptives. Concurrently, the urinary excretion of formiminoglutamic acid (FIGLU), a folate catabolite, was reported to increase.

Recent studies have focused on the relationship

between alcohol intake and folate nutriture. In this respect, Russell et al. (1983) indicated there is an increase in the urinary excretion and turnover time of folic acid associated with ethanol ingestion. While the exact mechanism has not been fully elaborated, many theories have been proposed. One theory advanced by Wilkinson and Shane (1982), holds that there is no effect on the hepatic or plasma total folate of the ethanol-fed rat. However, there is an increase in the rate of polyglutamate synthesis leading to an increase in tissue storage. The net result of which is a drop in the serum folate levels. A similar theory also was advanced by Hillman and Steinberg (1982), who suggested an alcohol-induced diversion of labeled folates into an intracellular polyglutamate pool. A somewhat different scenario was recently proposed by Halsted (1984). Chronic alcoholism was related to an impairment of the initial uptake of folates by the liver. The immediate result would be increased urinary wastage, while long-term folate metabolism is left unaffected.

Studies relating to other aspects of increased folate requirements have appeared. In one such study, Ek and Magnus (1982) suggested that formula-fed infants have an optimal folate intake of 75 µg/day during the first six months of life. Rogozinsky et al. (1983), who investigated the problem of folate nutrition in early pregnancy,

reported an intake range of 64.7 to 302.0 µg/day. The lowest intakes were associated with a maternal age of less than 20 years and with vomiting for 3 or more days a week. Finally, contrary to earlier reports, Swainson and Winney (1983) indicated there was no need for folate supplementation in patients on hemodialysis if they eat an adequate mixed diet. Research on the circumstances of increased folate needs are still far from being complete. In the meantime, the daily intakes of folacin as recommended by the WHO (1972) or the NRC-NAS Food and Nutrition Board (1980) should provide safe guidelines for the public as a whole. These recommendations are presented in Table 4.

Folate Fortification

It is now clear that the incidence of folate deficiency is high in many parts of the world. In most instances, it has been related to suboptimal nutrition occurring in conjuction with conditions of increased demands such as pregnancy and lactation. One approach to remedy this problem is to fortify certain foods with folates. A series of studies were conducted in this respect by Colman et al. (1974, 1975). Their findings suggest that folate deficiency during pregnancy can be prevented by fortifying a maize meal with pteroylglutamic acid in certain parts of the world. The meal used contained 300 ug of added folic acid and was

Table 4. WHO recommended NRC-NAS Food & Nutrition daily intakes of folates.

WHO recommendations		NRC-NAS Food & Nutrition		
Age or Status	Total folates (µg/day)	Group	Age years	Folacin (µg/day)
0- 6 months	40-50	Infants	0- 1	50
7-12 months	120	Children	1- 3	100
1-12 years	200		4- 6	200
>13 years	400		7-10	300
Pregnancy	800	Males	>11	400
Lactation	600	Females	>11	400
		Pregnancy		800
		Lactation		600

administered daily to subjects in late pregnancy. The practicality of their folate fortification policy was investigated in a rural Negro population of South Africa where folate deficiency occurred in 43.8% of nonanemic women in late pregnancy. The results of this series of studies indicated a need to consider food fortification with folate in similar populations. The hazards of such a policy are considered negligible. Similar conclusions were reached later by Thenen (1982), when she analyzed the folacin content of supplemental foods for pregnancy. Finally, and on a broader level, Colman (1982) suggested the addition of folic acid to staple foods as a selective nutrition intervention strategy.

CHAPTER III

MATERIALS AND METHODS

Experimental Design

The livers of two freshly slaughtered cattle were obtained from the Animal Science Department of the Louisiana State University. Each was immediately cut into 18 pieces approximately 1.5 cm thick. Four additional pieces were separated from each liver to be used in studies on % recovery and extract stability. The statistical design employed in this study was formulated in cooperation with the Experimental Statistics Department. A Randomized Block Design with a 3 x 2 x 3 factorial arrangement of the respective treatments was employed. Treatments consisted of three methods of preparation (raw, broiled or fried), two methods of packaging (vacuum and non vacuum) and three periods of frozen storage (30, 60 and 90 days). After the initial cooking treatment, all samples were cut into two pieces of approximately equal sizes: one was to be used as a control and the other was to be subjected to further treatment. All "control" samples and samples to be stored under vacuum (V) were immediately vacuum packed in polyethylene bags and stored protected from light at -20°C until analyzed. All other samples were stored under the same conditions except that they were not packaged under vacuum (NV).

All "control" samples were taken from the freezer and their folates extracted not later than 12 hours after being processed. Other samples were analyzed at periods of 30, 60 and 90 days of frozen storage.

Folate Extraction

Liver samples were divided into 1.5 cm pieces and 40.0 g homogenized in an Osterizer with 200 ml of 0.1M sodium acetate with 2% ascorbate (final pH adjusted to 4.5). A 50 ml aliquot of each homogenate was transferred to a graduated Sarstedt centrifuge tube and nitrogen gas bubbled into the homogenate for 30 sec. Each tube was then tightly closed and incubated for 3 hrs in a water bath at 37°C. The incubation period was employed to allow the endogenous conjugases to hydrolyse the folylpolyglutamates into monoglutamate forms which would be detectable by the HPLC procedure. For each sample, a duplicate homogenate was prepared and immediately frozen at -80°C.

Following incubation, the tubes were put into a water bath maintained at 95°C for 5 min in order to inactivate enzymes that might cause degradation or interconversions of the individual folates.

The heat treated homogenates were immediately cooled on ice for 20 min and then centrifuged at 2500 x g for 15 mins with a Diamon/IEC Division Cu-5000 centrifuge. The supernatant was decanted into the barrel of a 25 ml syringe and filtered through a 0.22 um Millipore filter unit.

The filtered extract was applied to a column of SM-2 Bio-Beads previously equilibrated with 0.1M sodium acetate. The column was washed with an additional 20 ml of the sodium acetate before final elution of the folates in 20 ml of 0.1M potassium phosphate buffer containing 2% ascorbic acid and 10% acetonitrile (final pH adjusted to 7.0). The extract from each sample was collected as four portions of 5 ml each into sterile Fisherbrand polypropylene tubes.

Nitrogen gas was bubbled into each tube for a period of 30 sec and the tubes were tightly closed and wrapped in aluminum foil. The tubes were further grouped according to their storage periods and each group was vacuum packed and stored in a Revco Ultra Low ultrafreezer at -80°C until further analyzed. All samples were analysed within a 90-day period.

Stability and Recovery Studies: Two samples were randomly selected from each liver and treated as all other extracts, in order to assess folate stability during frozen storage. They were analyzed at periods of 0, 30 ,60 and 90 days by the HPLC procedure, to be described later.

The effectiveness of the extraction procedure was evaluated by homogenizing three 40.0 gram samples of liver (raw, broiled or fried) in 200 ml of acetate-ascorbate. Aliquots (50 ml) of homogenate were taken in duplicate and treated like all other samples. Of the remaining 100 ml, two 25 ml portions were made to 50 ml each with 25 ml of a

1x[1] mixture of standards. Controls and treatments were subjected to the same extraction procedure described above. The percent recovery for each of the compounds under study was calculated by the following formula:

$$\frac{\text{(ng in trt tube - ng in control tube) x 100\%}}{\text{ng in 1x mixture}}$$

HPLC Analysis of Standards

Chromatographic conditions. A Varian Model 5000 Liquid Chromatograph was used throughout the study. The hydraulic components consisted of a single piston reciprocating pump driven by a 200 pulse/revolution stepper motor, three proportioning valves, flow controller and a pulse dampener to eliminate flow pulsations. A Varian's UV-10 ultraviolet detector, a Model 9176 Varian Recorder and a CDS 111 Integrator were employed also. All samples were injected with a 50 ul injection loop. A reverse-phase chromatography system adapted from a research paper by Day and Gregory (1981) was used. Faster separation was achieved by eliminating the use of coupled columns and derivatization. An Altex Ultrasphere IP (4.6mm x 25cm) preceded by a Whatman Standard Type Precolumn was equilibrated with a mobile phase consisting of 0.033M potassium phosphate pH 2.3 and 10% acetonitrile. The flow

[1] The 1x mixture of standards consisted of the following volumes of the original solutions: 4 ml pt-6-COOH, 2 ml p-ABG, 3 ml THF, 4 ml 5-CH3 -THF, 2 ml 5-CHO-THF, 6ml DHF and 4 ml FA. The final volume was made up to 250 ml.

rate was maintained at 1.0 ml/min with a resulting pressure of the order of 154 atm. Detection of the individual folates was achieved at room temperature at a wavelentgh of 280 nm. The detector's attenuation was set at 0.016 AU/mV.

Standards. Standards of pterin-6-carboxylic acid (Sigma P7279), p-aminobenzoylglutamic acid (Sigma A0879), tetrahydrofolic acid (Sigma T3125), 5-methyltetrahydrofolic acid as sodium salt (Sigma M0132), dihydrofolic acid (Sigma D7006), 5-formyltetrahyrofolic acid (Sigma F7878) and pteroylglutamic acid (Sigma F7876) were received under cool dry conditions and were immediately transferred to a dessicator protected from light and maintained at -20°C until further use. Standards of pterin-6-carboxylic acid and pteroylglutamic acid were initially dissolved in a 5% w/v pH 9.1 dibasic potassium phosphate and quickly diluted in a protective 0.1M potassium phosphate buffer containing 2% ascorbate and 10% acetonitrile (pH adjusted to 7.0). All other standards were dissolved directly in the phosphate ascorbate buffer. Stock solutions of the individual standards were prepared by incorporating into 100 ml of buffer each of the following: 5 mg of pt-6-COOH, 20 mg of p-ABG , 20 mg of 5-CHO-THF, 5 mg of 5-CH3-THF, 20 mg of THF, 10 mg of DHF and 10 mg of FA. A range of dilutions of each standard were injected into the system until a satisfactory peak was obtained. The appropriate concentrations were found to be 125 ng of pt-6-COOH, 200 ng

of p-ABG, 250 ng of 5-CHO-THF, 100 ng of 5-CH3-THF, 300 ng of THF, 400 ng of DHF and 125 ng of FA.

Mixture of standards. To prepare a mixture of standards reflecting the same concentrations of the individual standards from the stock solutions, the following volumes were used: 1.0 ml of pt-6-COOH, 0.4 ml of p-ABG, 0.5 ml of 5-CHO-THF, 0.4 ml of 5-CH3-THF, 0.3 ml of THF, 0.8 ml of DHF and 0.5 ml of FA. The mixture was brought to a final volume of 20 ml with the phosphate ascorbate buffer.

Reproducibility, linearity and minimum detectable amounts. To check the reproducibility of the results, five injections for each of the standards and the mixture of standards were run and the results analyzed for respective retention times and peak heights. Linearity was assessed by injecting different dilutions of the same standard or of the mixture of standards and plotting peak heights vs the quantity injected. In each case, at least 5 points were plotted corresponding to 1/4, 1/3, 1/2, 1/1 and 2/1 of the respective original dilutions of each standard. Regression analysis on all sets of readings established a linear relationship between peak height and quantity of standard injected. Finally, successively lower dilutions of each of the standards were injected until it was no longer possible to detect the standard at the appropriate attenuation. The amount of standard determined from the next higher dilution

was considered the minimum detectable level.

HPLC Analysis of Liver Folates

Frozen extracts for the HPLC analysis were thawed for 10 min at 37°C in the dark. They were filtered thru a 0.22 um Millipore filter and approximately 200 µl was transferred to a syringe and manually injected in the system through the 50 µl sample loop. Operating conditions were maintained as described for the chromatography of standards. A 20 min column stabilization period was used between each run. After each 10 injections, an external standard was run and compared to the original chromatogram mixture of standards. Quantification of the individual folates was achieved by matching retention times and peak heights. Triplicates were averaged for each sample. Any value falling outside the 95% confidence limits was discarded from the analysis.

Microbial Analysis

Organism rehydration. A pure, lyophilized culture of Lactobacillus casei (ATCC 7469) was obtained through Difco (Detroit, Michigan) and stored at 5°C until further use. A rehydration medium consisting of 100 g of non-fat dry milk, 5 g Difco yeast extract and 100 ml of filtered canned tomato juice was mixed under heat and made up to a 1 liter volume with distilled water. The pH of the medium was brought to 6.8 with 5% NH_4OH before autoclaving for 15 min

at 121°C under 15 psi. The rehydration medium was cooled to room temperature and 0.5 ml was aseptically transferred to the vial that contained the L. casei culture. This provided a uniform dissolution of the lyophilized culture prior to its aseptic transfer into the rehydration medium, which was then lightly shaken to achieve a homogeneous suspension. Ten ml of the rehydration culture was asepticaly poured into each of four sterile 15 x 150 mm screw-cap tubes which were then incubated at 37°C for 48 hours.

Following the incubation period, the rehydration culture was allowed to cool to room temperature and then used to make 8 stab cultures. Stab cultures consisted of 15 x 150 mm screw-cap test tubes each containing 10 ml of sterilized solidified Micro Assay Culture Agar (Difco 0319-01), inoculated by stabs from the rehydration culture and incubated at 37°C for 18 hr. The stab cultures were kept refrigerated at 5°C until needed. Prior to each assay, the culture was revived by transferring for two consecutive days in a similar medium and incubating at 37°C.

Inoculum. The microorganism was inoculated with a borosilicate loop into two 18 x 160 mm test tubes each containing 10 ml of Elliker Bacto Broth (Difco 0974-01-1) and incubated at 37°C for 18 hr. The tubes were then centrifuged for 20 min at 2500 x g. The supernatant was decanted and 10 ml of sterilized 0.85% saline was

aseptically added to the culture. The tubes were gently vortexed to achieve a uniform suspension and centrifuged again under the same conditions. This washing procedure was repeated three times, at the end of which the organism was aseptically suspended in 10 ml of sterile distilled water. A further dilution was achieved by suspending 0.1 ml in 10 ml of sterile distilled water. One drop of this preparation was used to inoculate each tube of both the standards and the samples.

To prepare the standard folic acid solution, 200 mg of crystalline folic acid (Sigma F7876) was dissolved in 1000 ml of 20% absolute ethanol in 0.01N NaOH. This stock solution, containing 200 ug of folic acid/ml, was divided into aliquots of 3 ml each and stored at -80^{o}C until further use. One ml of the stock solution was diluted 1:100 three consecutive times in the actual assay. This resulted in a standard solution which contained 0.2 ng of folic acid per ml. This working solution was added to a series of 18 X 200 mm test tubes as listed in Table 5.

Table 5. Sample dilution for folic acid (F.A.)

Tube # (triplicate)	ml Standard solution/tube	ng Folic Acid per tube
Blank	0.0	0.0
0	0.0	0.0
1	0.5	0.1
2	1.0	0.2
3	1.5	0.3
4	2.0	0.4
5	2.5	0.5
6	3.0	0.6
7	3.5	0.7
8	4.0	0.8
9	4.5	0.9
10	5.0	1.0

The tubes were made to a volume of 5.0 ml with 0.15% ascorbate solution (pH 6.1) and then to 10.0 ml with Folic Acid Casei Medium (Difco 0822-15-9). All tubes were subjected to the same analytical protocol which was applied to the samples and a new standard curve was prepared for each assay.

Test Samples were taken out of the ultrafreezer and brought to room temperature in the dark. A 1 ml aliquot was extracted from each tube and diluted by a factor of 1:5000 (1:100 then 1:50) with the ascorbate solution. For each sample, 5 successive dilutions were prepared in duplicates ranging from 1 to 5 ml. All tubes were made to 5 ml with the ascorbate solution and then to 10 ml with the Folic Acid Casei Medium . Standard and sample tubes were autoclaved for 5 min at 121^{o}C and 15 lbs of pressure,

allowed to cool to room temperature in a water bath and then inoculated. Following inoculation they were incubated in a covered water bath set at 37°C for 18 hr. No more than 10 samples were assayed during each run and all operations were performed in the absence of artificial illumination and minimal natural light.

At the end of the incubation period, all tubes were transferred to a cooler and maintained at 5°C to prevent further microbial growth. A Spectronic-25 spectrophotometer was used to determined turbidity by monitoring absorption at 640nm. A linear regression model was obtained from the standard solution and a standard curve plotted for each assay. Folate concentrations in test samples were determined by fitting absorbance values into the regression formula. Absorbances which fell outside of the 95% confidence limits of the standard curve were excluded from the average calculation. Folate concentrations were then expressed as µg/100 g of liver on both a wet and dry weight basis.

CHAPTER IV

RESULTS

HPLC Analysis of Standards and Mixtures

The retention times and peak heights obtained from the HPLC analysis of folates are presented in Table 6.

Table 6. Retention times and peak heights obtained from the analysis of folates.

Standard	Concentration (ng)	Retention time (min)	Peak height (mm)
pt-6-COOH	125	3.16	152
p-ABG	200	3.84	179
THF	300	5.64	131
5-CH_3-THF	100	6.76	115
5-CHO-THF	250	16.03	50
DHF	400	18.63	139
FA	100	22.54	45

A linear relationship between concentration and peak height was established for all standards over a five fold range. A mixture of the standards was successfully separated under the same chromatographic conditions and is shown in Figure 5. A representative chromatogram of a liver extract is shown in Figure 6.

Fig. 5--Chromatographic separation of a standard mixture of folic acid derivatives and oxidation products.

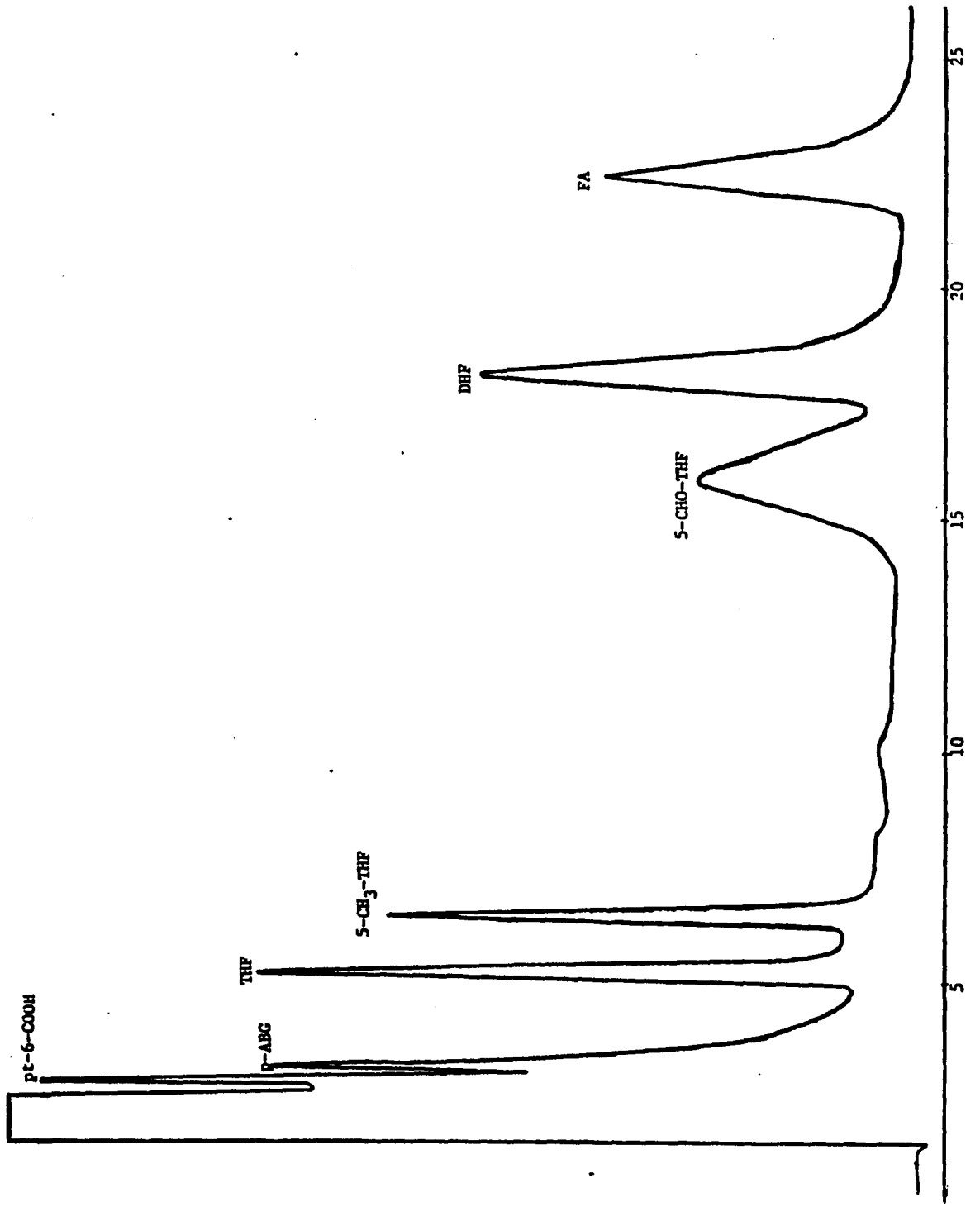
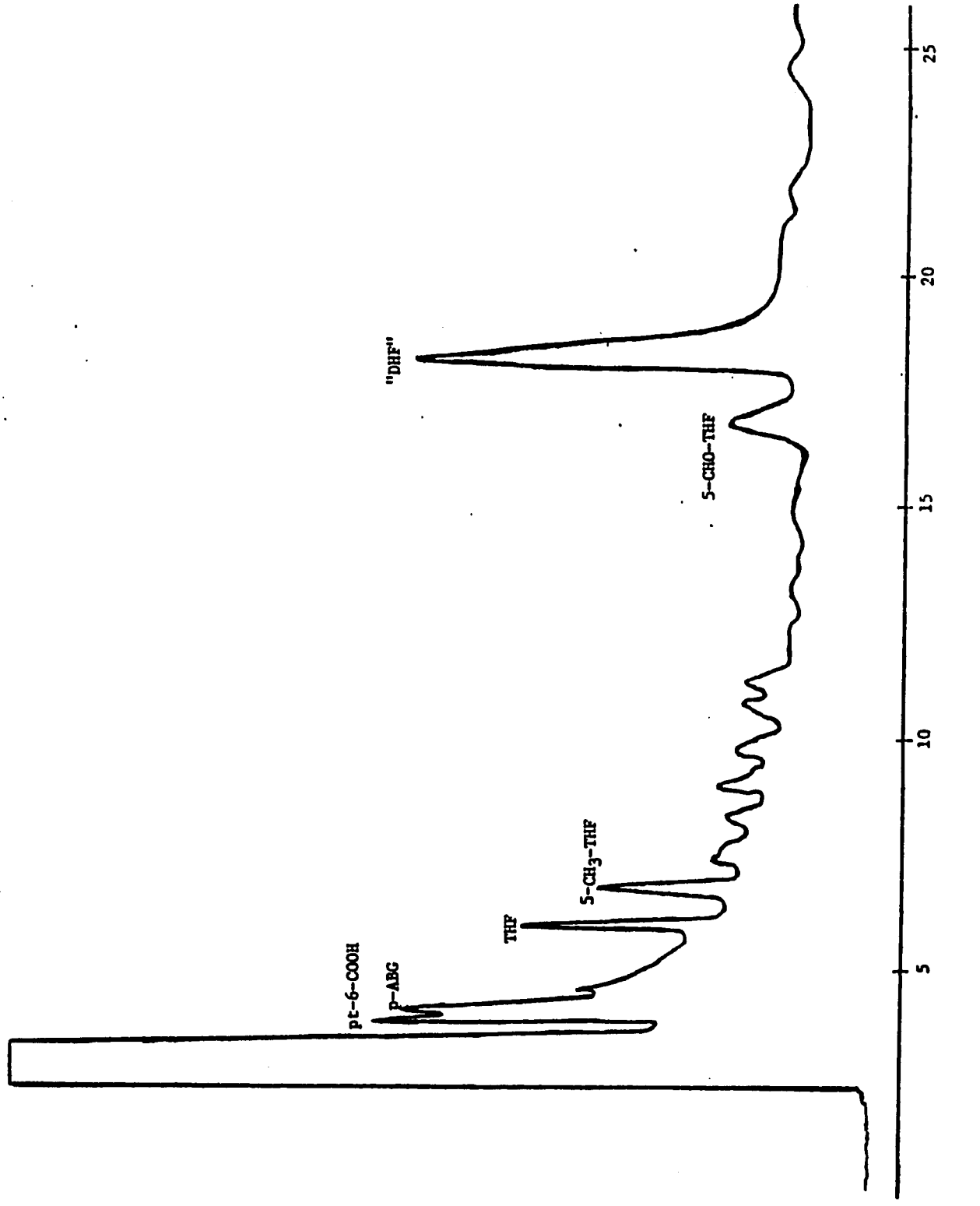


Fig. 6-Chromatographic separation of folic acid derivatives and oxidation products in beef liver.



HPLC Analysis of Folate Extracts

Stability and recovery studies. No significant change ($p<0.01$) in the folate profile of liver extracts was detected upon storage for 90 days at -80°C in the dark (Appendix A). The results of the analyses of samples designated for recovery studies are presented in Table 7.

Table 7. The recovery rate (%) of folates from liver samples.

Product	% Recovery		
	Raw	Broiled	Fried
pt-6-COOH	87	85	85
p-ABG	90	87	85
5-CH_3-THF	82	83	82
THF	72	70	76
5-CHO-THF	81	84	79
DHF	87	85	83
FA	89	84	86

Folate derivatives and oxidation products. The raw data pertaining to the quantitation of the different folate derivatives and oxidation products can be found in Appendices A through F. The ANOVA's of the variables under study, relating to the difference between treatments and controls, are presented in Tables 8 - 13.

Table 8. Analysis of variance for pt-6-COOH

SOURCE	DF	TYPE I SS	F VALUE	PR > F
REP	1	133590.25	1.40	0.2533
COOK	2	345740.67	1.81	0.1939
STORE	1	971867.36	10.17	0.0054*
COOK*STORE	2	713099.56	3.73	0.0453*
TIME	2	3440213.17	18.00	0.0001*
COOK*TIME	4	2365856.67	6.19	0.0029*
STORE*TIME	2	255100.06	1.34	0.2894
COOK*STORE*TIME	4	731100.78	1.91	0.1545

Table 9. Analysis of variance for p-ABG

SOURCE	DF	TYPE I SS	F VALUE	PR > F
REP	1	1897047.11	13.72	0.0018*
COOK	2	1593492.06	5.75	0.0123*
STORE	1	4164320.44	30.12	0.0001*
COOK*STORE	2	15851278.72	57.33	0.0001*
TIME	2	18284635.06	66.13	0.0001*
COOK*TIME	4	5124557.44	9.27	0.0004*
STORE*TIME	2	447002.06	1.62	0.2276
COOK*STORE*TIME	4	8751658.78	15.83	0.0001*

Table 10. Analysis of variance for THF

SOURCE	DF	TYPE I SS	F VALUE	PR > F
REP	1	31211.11	0.94	0.3468
COOK	2	748608.39	11.23	0.0008*
STORE	1	4268.44	0.13	0.7249
COOK*STORE	2	142581.06	2.14	0.1485
TIME	2	155232.39	2.33	0.1277
COOK*TIME	4	633378.28	4.75	0.0093*
STORE*TIME	2	102458.39	1.54	0.2435
COOK*STORE*TIME	4	760745.61	5.70	0.0042*

Table 11. Analysis of variance for 5-CH_3-THF

SOURCE	DF	TYPE I SS	F VALUE	PR > F
REP	1	1296.00	1.33	0.2644
COOK	2	92633.39	47.60	0.0001*
STORE	1	12996.00	13.36	0.0020*
COOK*STORE	2	58483.50	30.06	0.0001*
TIME	2	48759.05	25.06	0.0001*
COOK*TIME	4	97367.44	25.02	0.0001*
STORE*TIME	2	1953.50	1.00	0.3872
COOK*STORE*TIME	4	44737.00	11.50	0.0001*

Table 12. Analysis of variance for 5-CHO-THF

SOURCE	DF	TYPE I SS	F VALUE	PR > F
REP	1	69872.11	1.27	0.2759
COOK	2	737460.72	6.69	0.0072*
STORE	1	132253.44	2.40	0.1398
COOK*STORE	2	149132.39	1.35	0.2850
TIME	2	2476387.39	22.46	0.0001*
COOK*TIME	4	6574516.61	29.82	0.0001*
STORE*TIME	2	811570.06	7.36	0.0052*
COOK*STORE*TIME	4	1448868.61	6.57	0.0022*

Table 13. Analysis of variance for DHF

SOURCE	DF	TYPE I SS	F VALUE	PR > F
REP	1	9450500.69	6.07	0.0247*
COOK	2	13896864.00	4.47	0.0276*
STORE	1	16598834.03	10.67	0.0046*
COOK*STORE	2	20990060.22	6.74	0.0070*
TIME	2	43122107.17	13.86	0.0003*
COOK*TIME	4	33430626.83	5.37	0.0055*
STORE*TIME	2	7888542.06	2.53	0.1088
COOK*STORE*TIME	4	43227273.94	6.94	0.0017*

In all the above ANOVA tables, the asterisk denotes statistically significant results at $p < 0.05$. These results indicate that the replicate used was a significant factor affecting the changes in both p-ABG and DHF. However, it should be noted that in the raw samples, the p-ABG and DHF contents of liver 2 were 22% and 104%, respectively higher than those of liver 1. The cooking treatment had a significant effect on all of the variables except for pt-6-COOH, while the type of storage (vacuum or non-vacuum) did not seem to influence THF and 5-CHO-THF levels. Also, THF was not affected by the duration of the frozen storage.

As for the interactive effect of different treatments, cooking by storage was significant for all variables except THF and 5-CHO-THF. Cooking by time effected all variables, and storage type by length of storage was only significant for 5-CHO-THF. Finally, the combination of cooking method by storage type and length of storage had a significant effect on all variables under study except pt-6-COOH.

On the other hand, the HPLC analysis revealed that regardless of the cooking treatment, pteroylglutamic acid (unreduced folic acid) was absent from all samples while dihydrofolate was possibly present at relatively high concentrations. This is illustrated in Table 14 listing the mean values for each variable in the controls by liver

and cooking method.

Table 14. Means of folate derivatives in controls

	pt-6-COOH	p-ABG	THF	5-CH_3-THF	5-CHO-THF	DHF	FA
	ug/100g dry basis						
R1	2396	3009	1359	407	1547	9140	0
R2	3560	3659	1343	496	1931	18691	0
B1	1571	3686	1161	194	472	2538	0
B2	2724	4180	1022	217	416	4082	0
F1	1349	3915	918	251	675	2657	0
F2	2812	4915	786	271	758	4955	0

R = Raw B = Broiled F = Fried

Differences in folates and their oxidation products due to the cooking treatments are graphically depicted in the histograms of Figures 7 - 12. The change in each of the variables, calculated as percent from the original value in the raw samples is presented in Table 15.

Table 15. Change in folates derivatives in controls due to cooking.

	pt-6-COOH	p-ABG	THF	5-CH_3-THF	5-CHO-THF	DHF
	%					
B1	66	123	85	48	31	28
B2	77	117	76	44	22	22
F1	56	130	68	62	44	29
F2	79	138	59	55	39	27

B = Broiled F = Fried

In general, the order of stability of the different folates towards cooking (whether broiling or frying) was as follows: "DHF" < 5-CHO-THF < 5-CH_3-THF < THF. As for the oxidation products, levels of p-ABG increased while those of pt-6-COOH unexpectedily decreased upon cooking.

The effects of package type and length of storage on the different variables are represented in Figures 13 - 18. The changes in concentrations, calculated as percent of the original value in the control samples are presented in Table 16.

Table 16. Changes in concentrations of folates as affected by type of heating.

Air	RAW			BROIL			FRY		
	30	60	90	30	60	90	30	60	90
	---------- % ----------								
pt-6-COOH	125	92	120	120	126	105	85	108	164
p-ABG	56	123	163	159	142	147	80	106	122
THF	35	70	46	75	24	37	75	49	41
5-CH_3-THF	31	41	95	112	62	141	74	59	38
5-CHO-THF	171	36	168	282	21	121	64	143	203
DHF	79	37	103	89	75	54	59	53	103
Vacuum	**RAW**			**BROIL**			**FRY**		
	30	60	90	30	60	90	30	60	90
	---------- % ----------								
pt-6-COOH	101	102	123	80	76	108	79	109	153
p-ABG	112	126	132	66	61	120	72	113	130
THF	60	46	28	66	20	28	61	44	63
5-CH_3-THF	75	77	100	72	74	68	102	63	96
5-CHO-THF	131	101	112	139	23	118	41	196	454
DHF	107	92	93	95	46	117	48	65	106

Fig. 7--Effect of cooking on pt-6-COOH

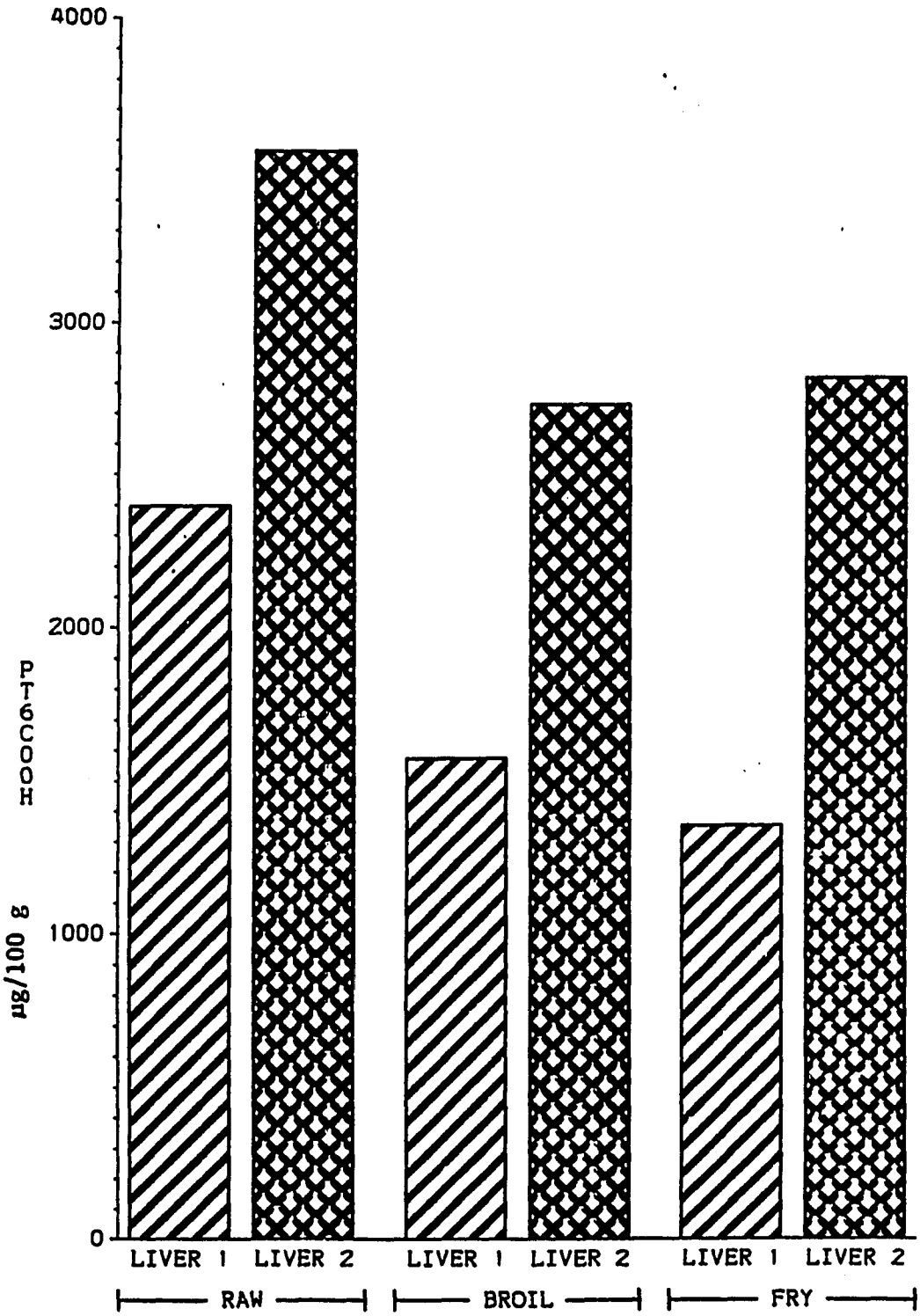


Fig. 8--Effect of cooking on p-ABG

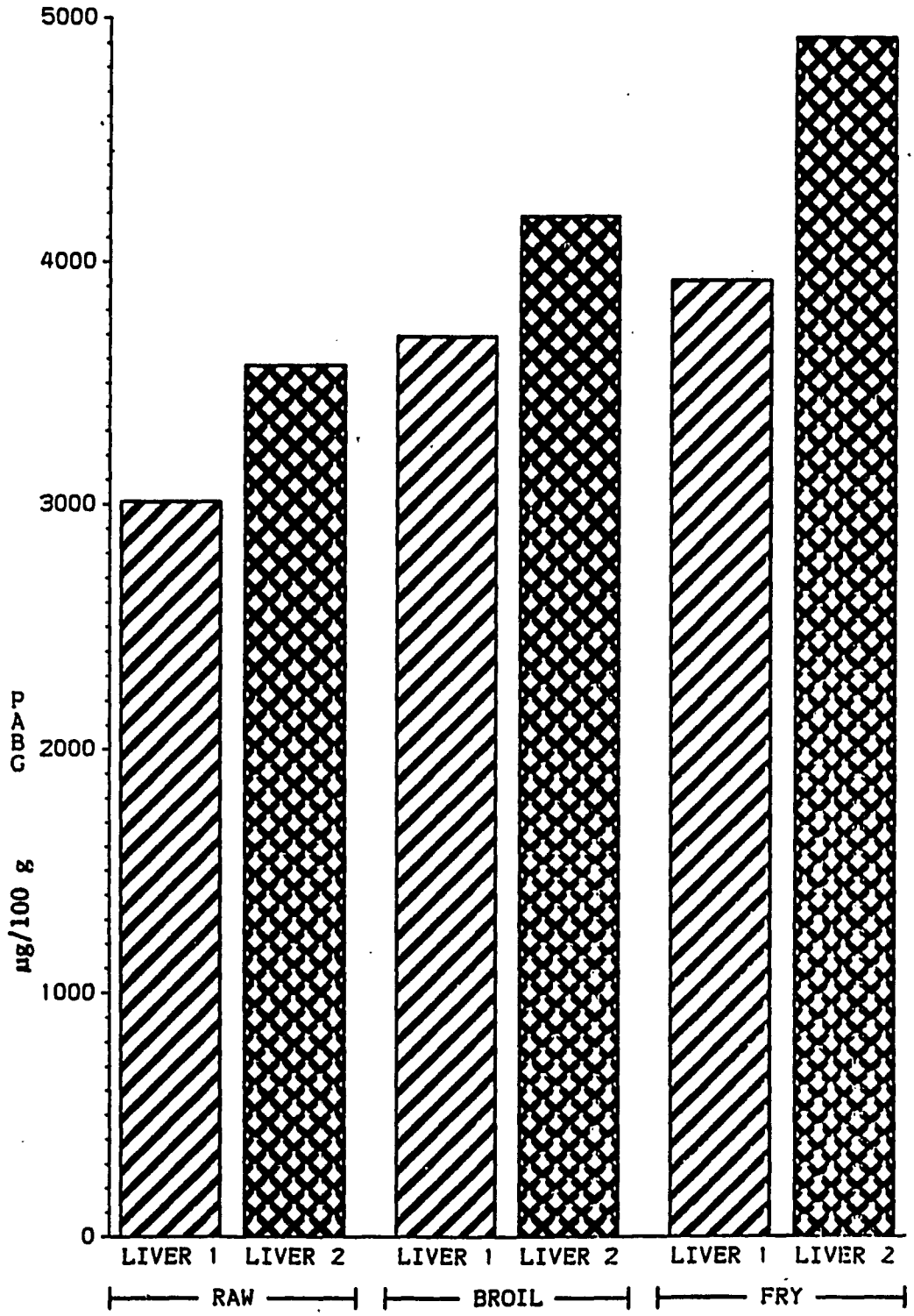


Fig. 9--Effect of cooking on THF

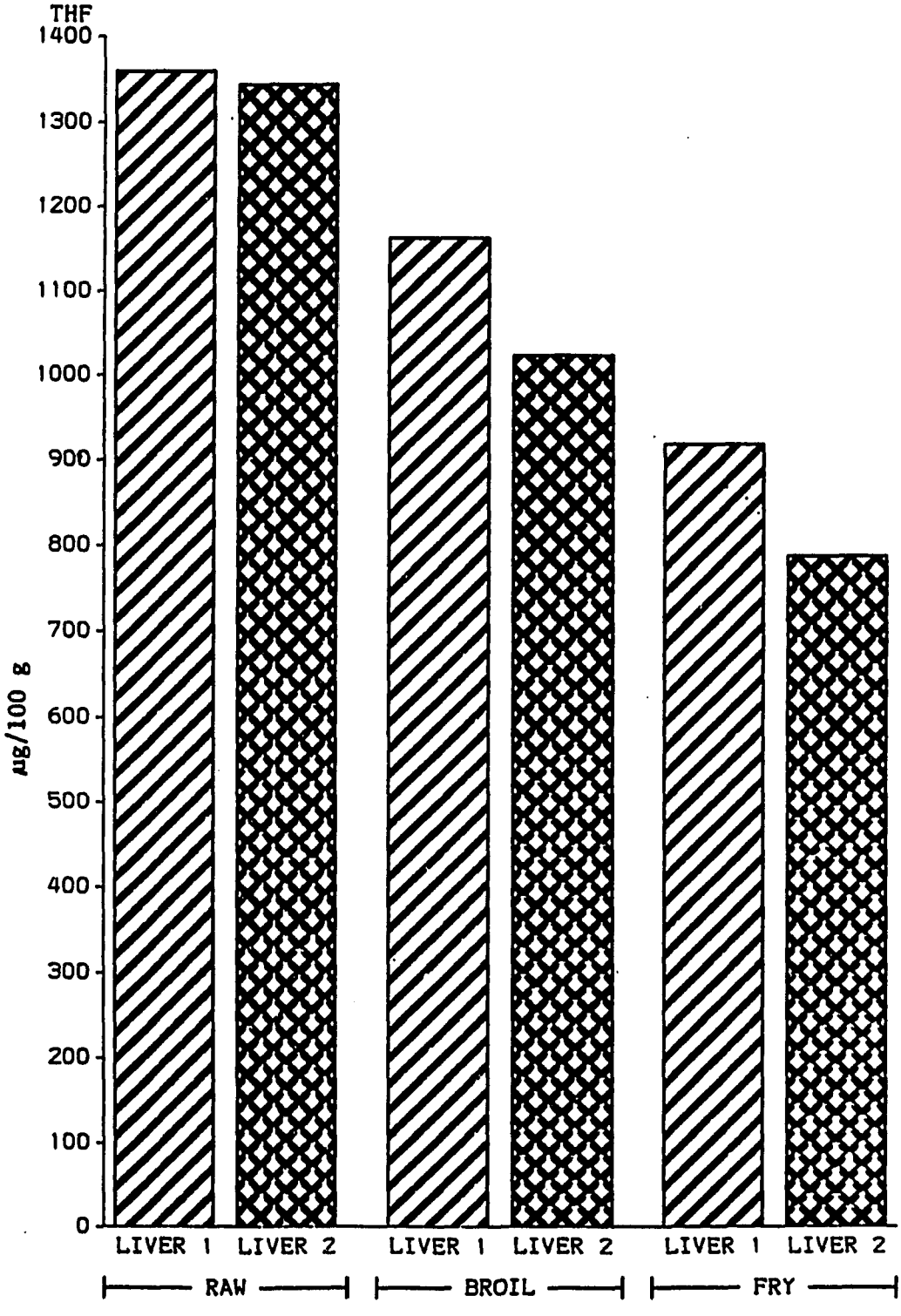


Fig. 10--Effect of cooking on 5-CH_3-THF

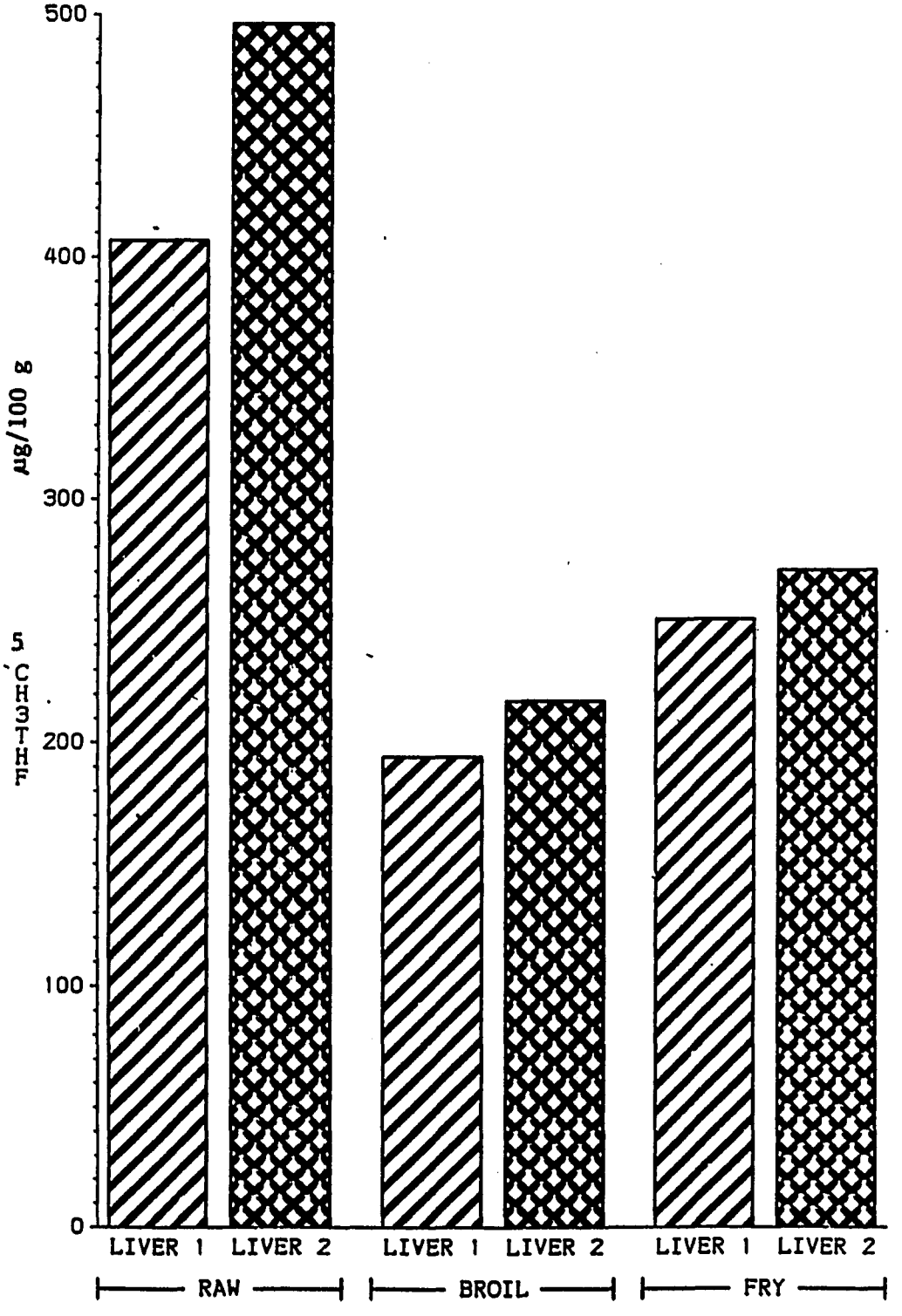


Fig. 11--Effect of cooking on 5-CHO-THF

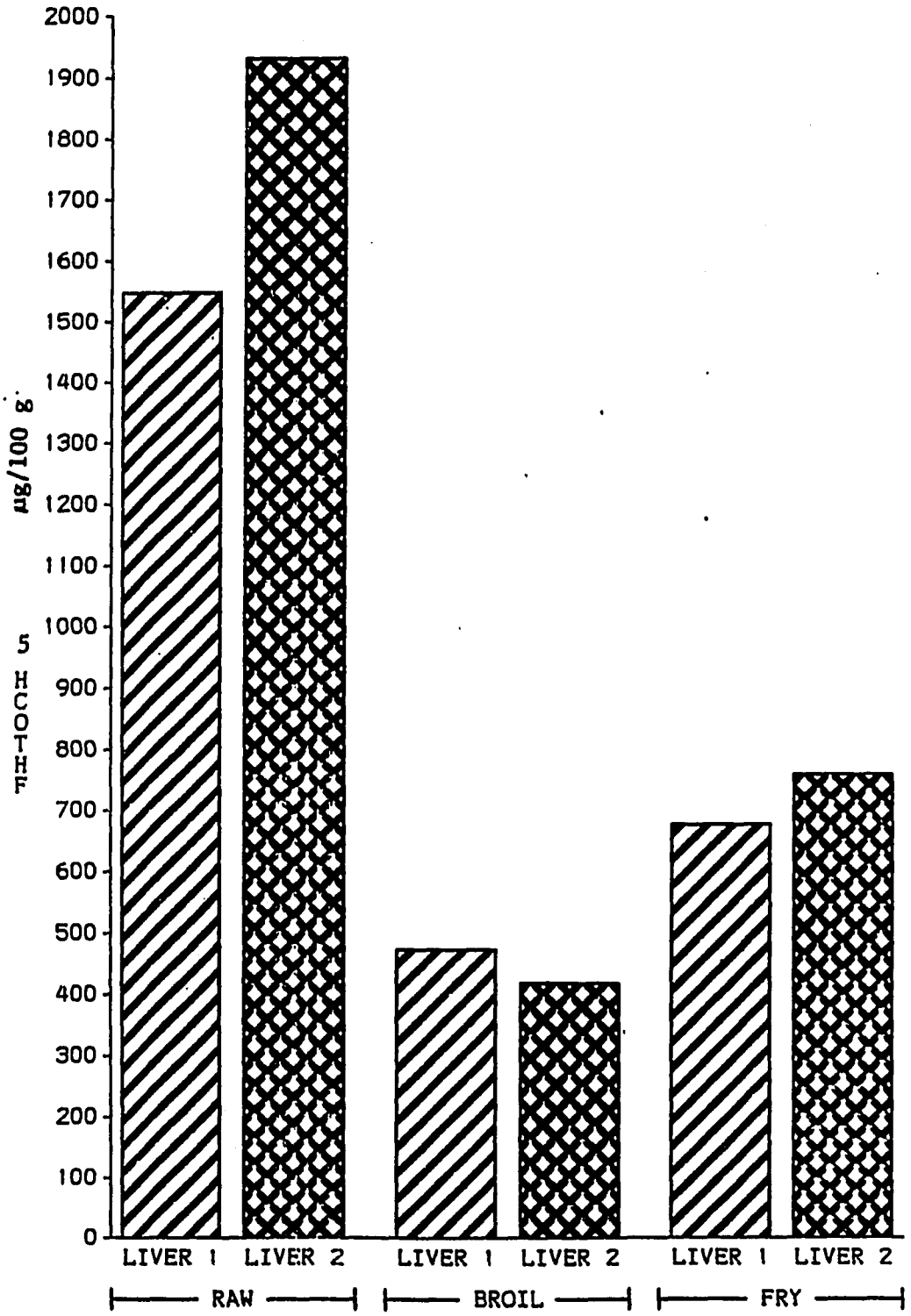


Fig. 12--Effect of cooking on DHF

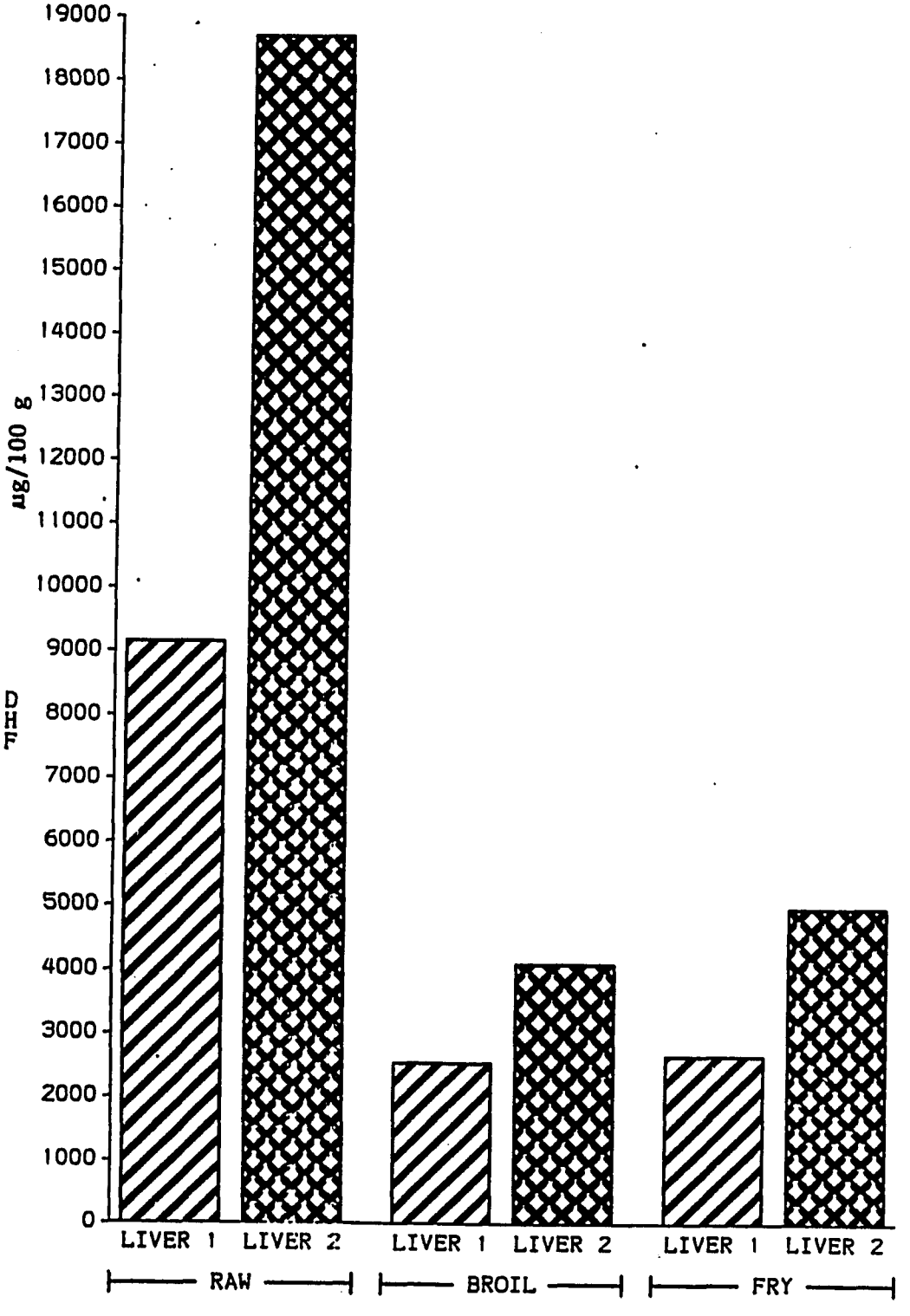


Fig. 13--Changes in pt-6-COOH during frozen storage for different cooking and packaging treatments.

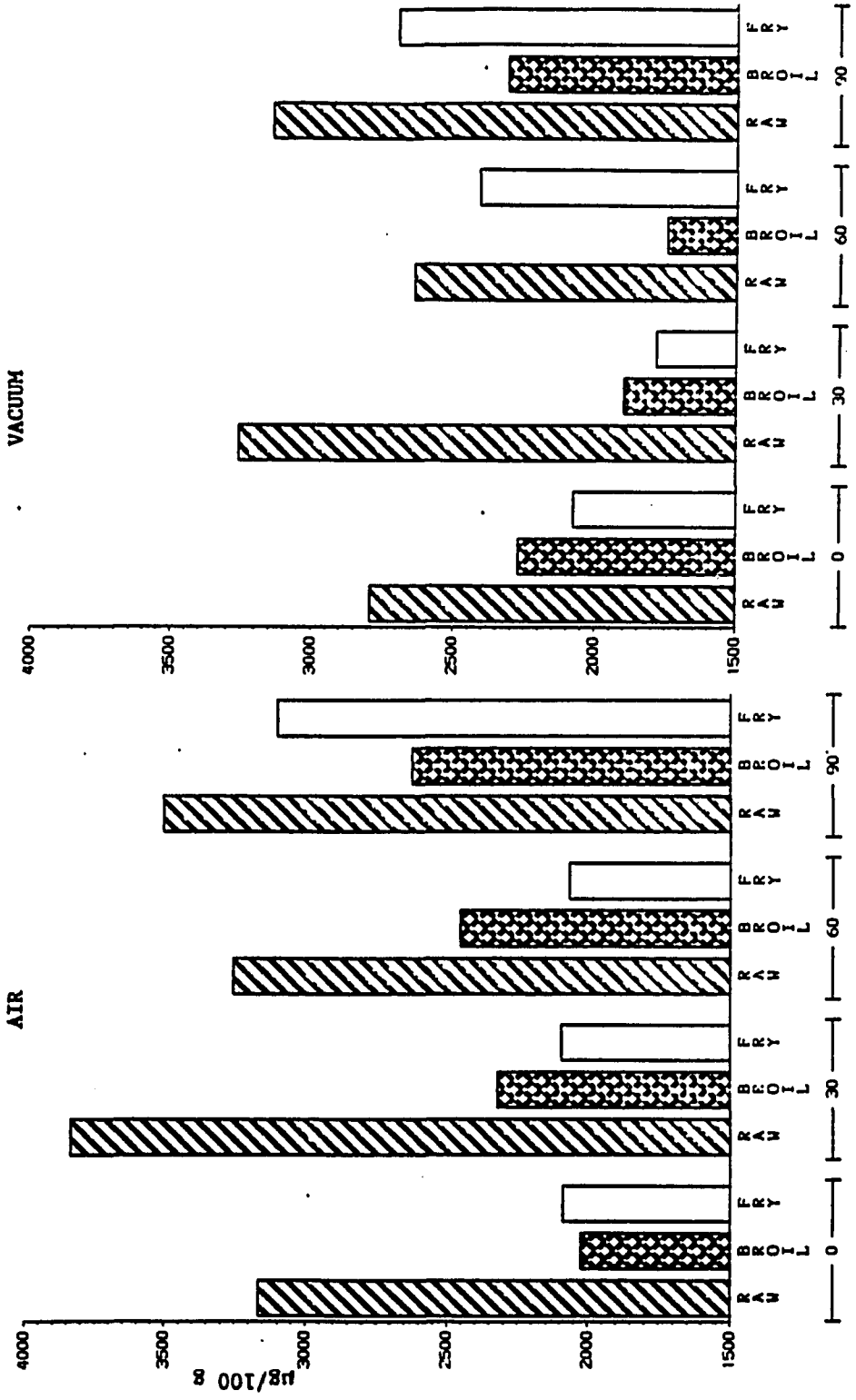


Fig. 14--Changes in p-ABG during frozen storage for different cooking and packaging treatments.

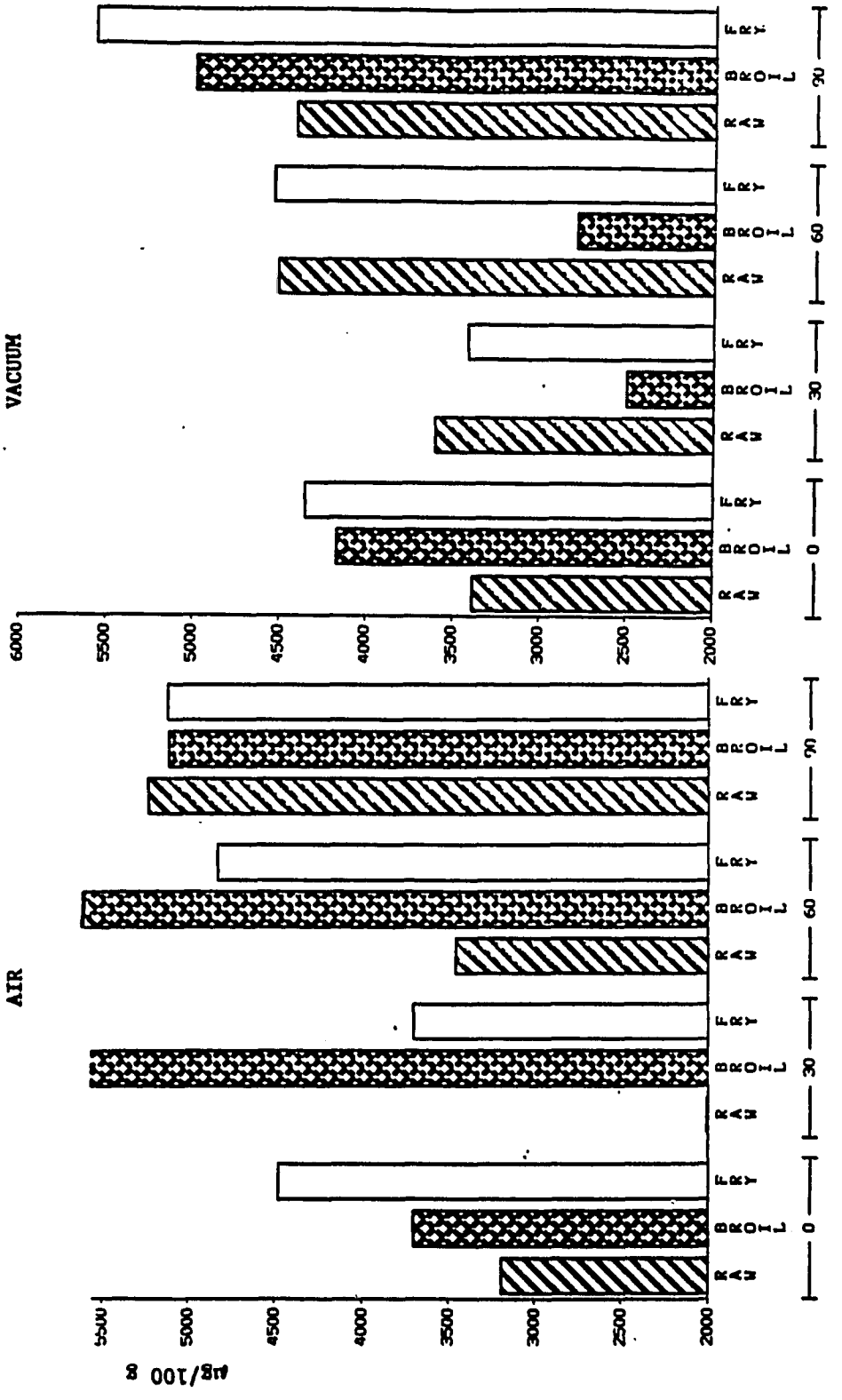


Fig. 15--Changes in THF during frozen storage for different cooking and packaging treatments.

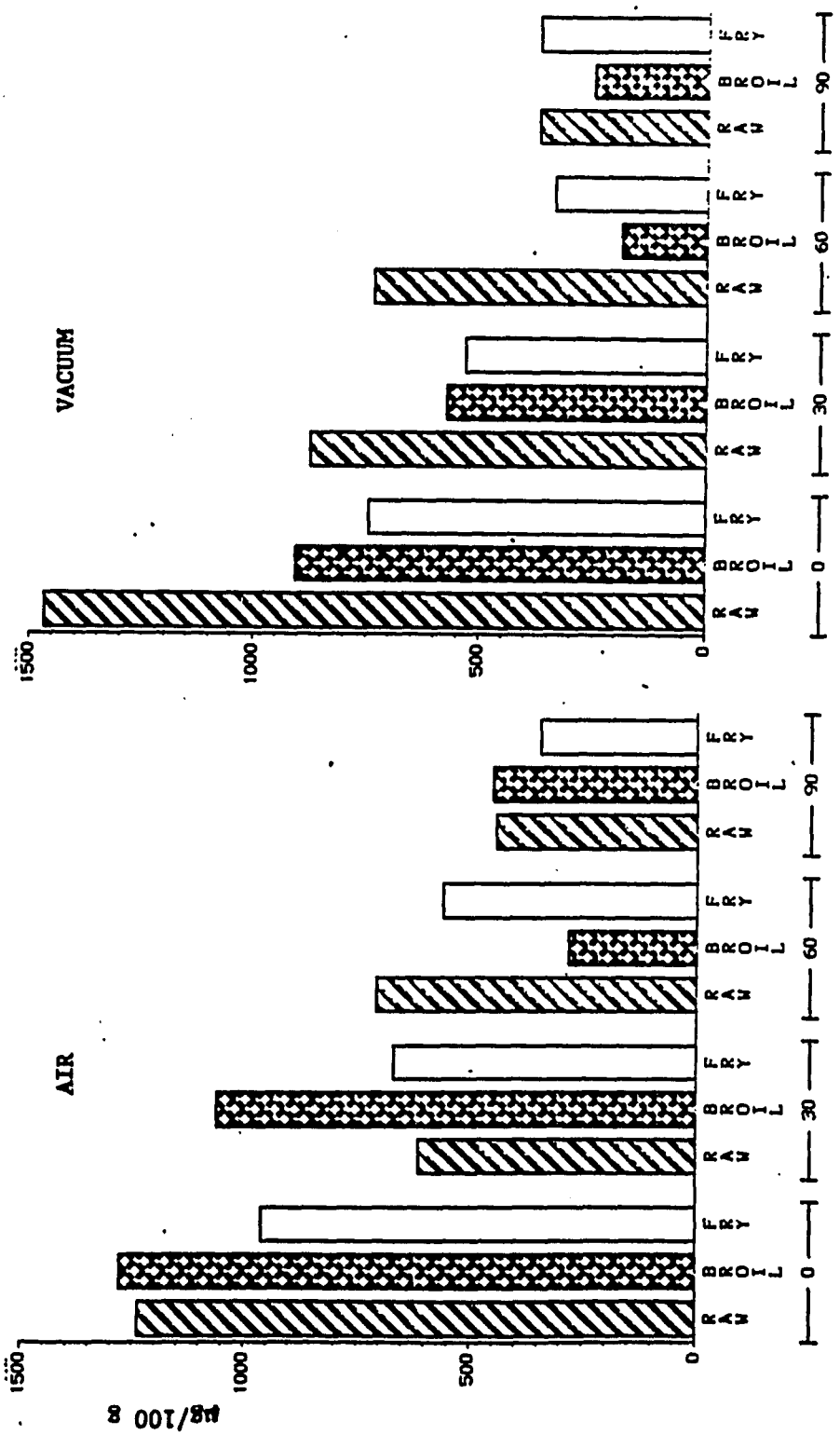


Fig. 16--Changes in 5-CH_3-THF during frozen storage for different cooking and packaging treatments.

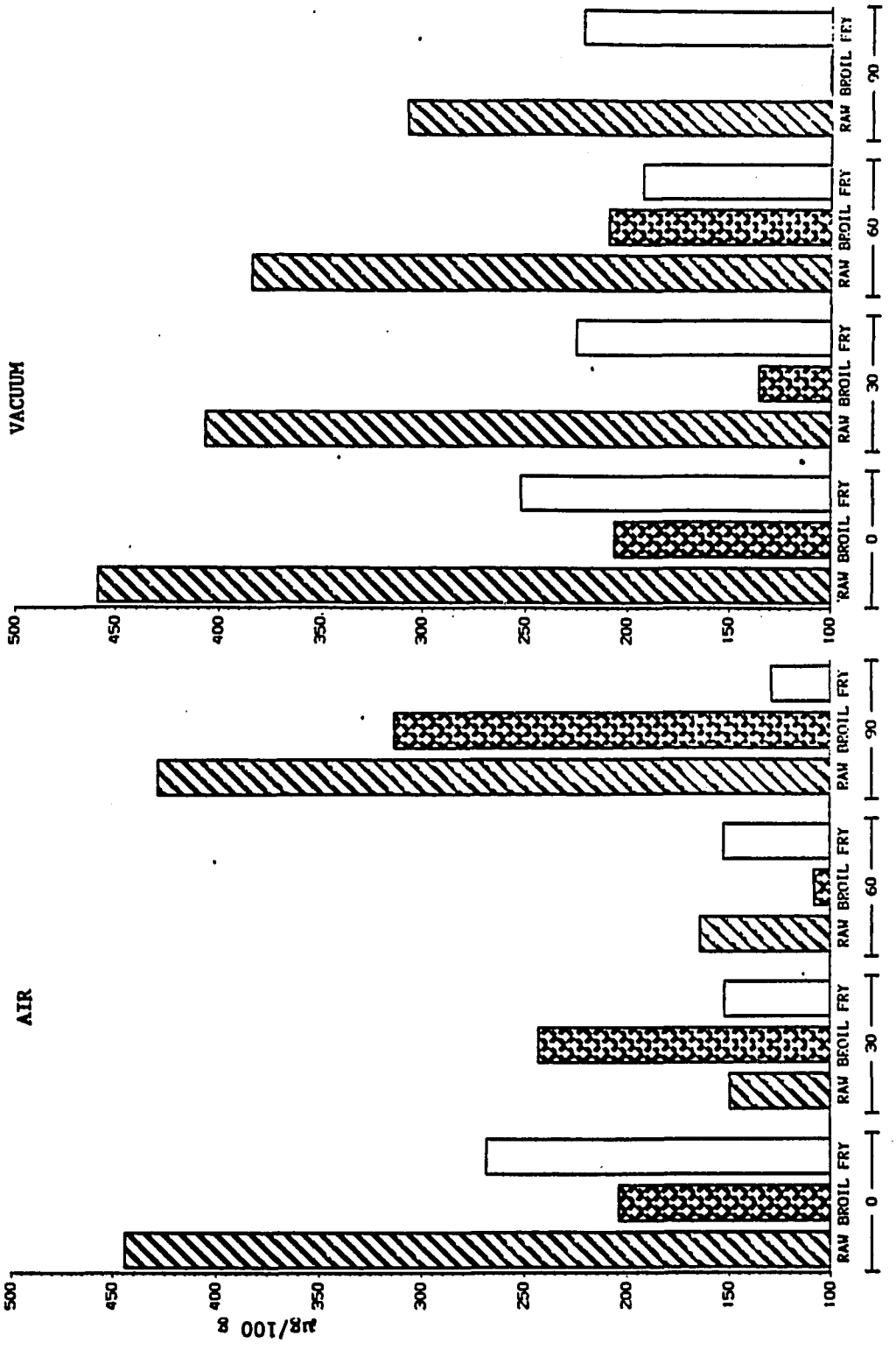


Fig. 17--Changes in 5-CHO-THF during frozen storage for different cooking and packaging treatments.

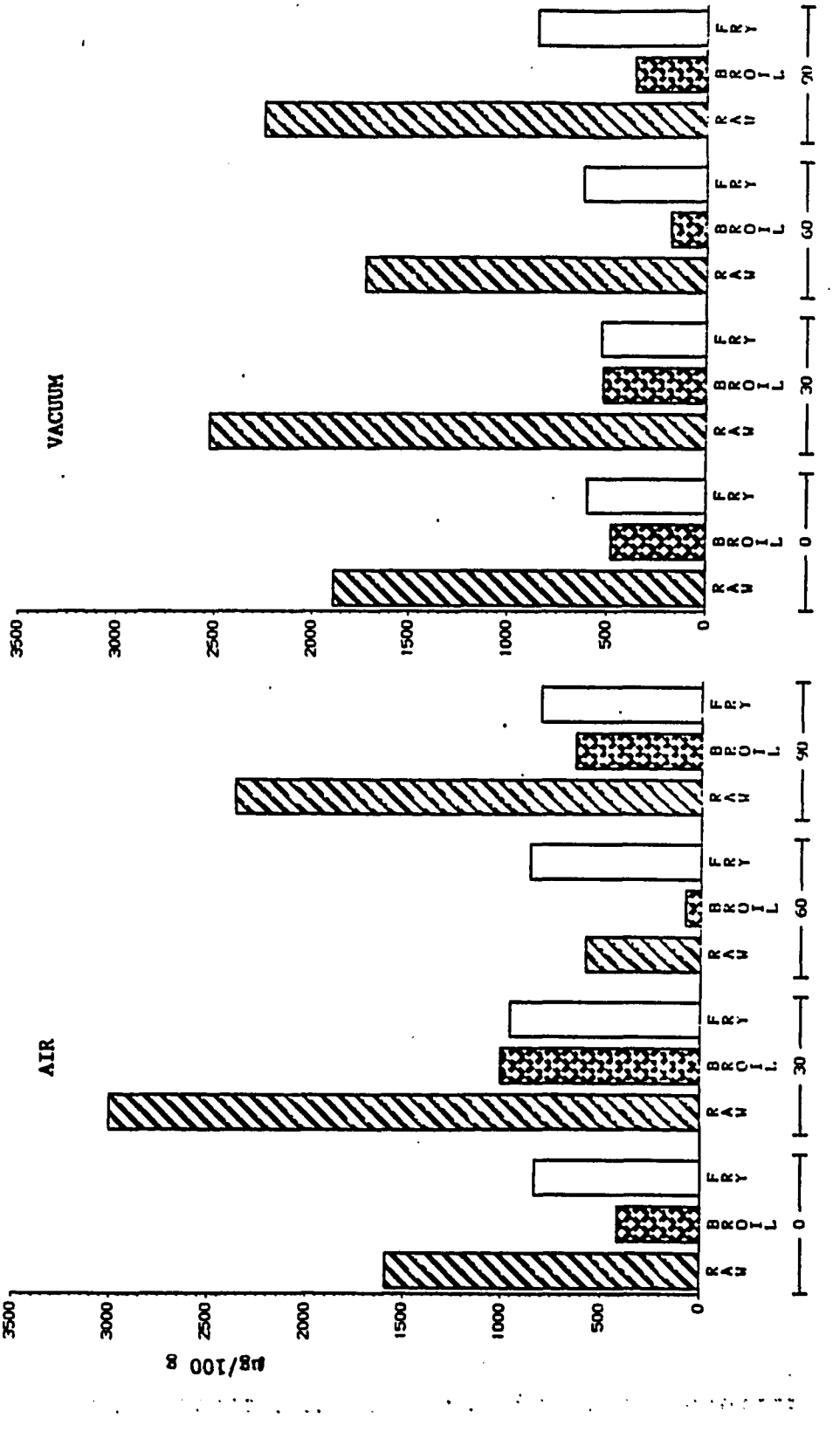
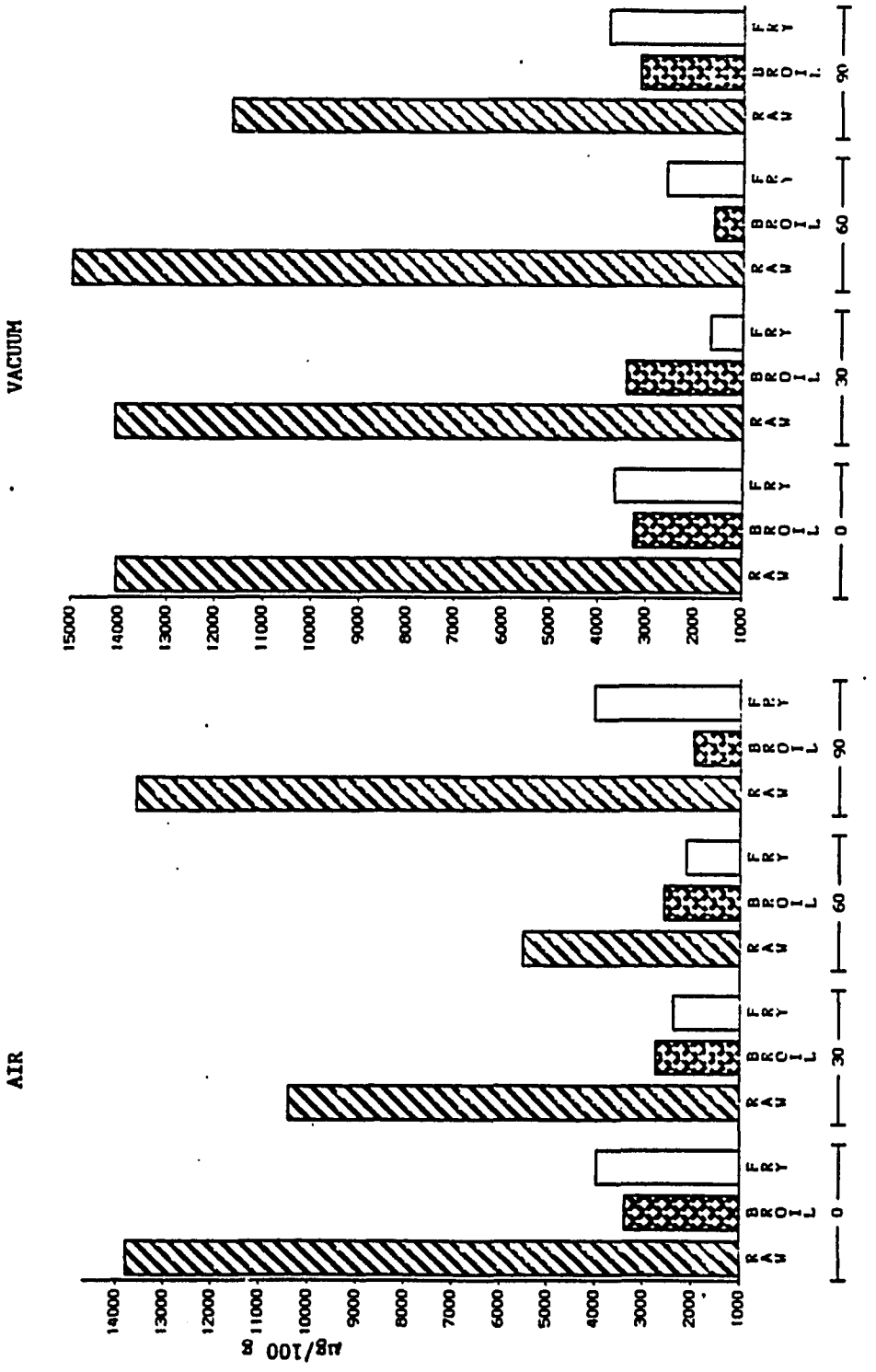


Fig. 18--Changes in DHF during frozen storage for different cooking and packaging treatments.



Irrespective of the cooking treatment, the increase in pt-6-COOH levels was lower after 30 days of frozen storage when vacuum packaging was used. However, packaging method did not seem to affect the changes in the concentrations of this oxidation product for a storage period of 90 days. The second oxidation product, p-ABG, increased twice as much when raw liver was stored under vacuum as opposed to no vacuum for 30 days. It behaved differently in broiled liver, however, where the change was greater in non-vacuum than in vacuum packed samples, whereas variations in p-ABG levels seemed to be independent of packaging method for fried liver.

As for the individual folates, levels of THF, "DHF" and 5-CH_3-THF generally decreased while those of 5-CHO-THF increased in either vacuum or non-vacuum packages. However, these trends were not always linear over time. In many cases, a folate which had originally dropped in concentration after 60 days of storage reappeared at higher concentrations after 90 days (e.g. 5-CH_3-THF, Fig. 16, Air).

Total folates. Since the high DHF content of liver extracts is a potential controversial subject, two values for "total folates by HPLC" will be reported. For the sake of convenience "Total Folates 1" will represent the sum of 5-CH_3-THF, THF and 5-CHO-THF, while "Total Folates 2" will include in addition values for DHF. The raw data for both

of these variables are found in Appendices H and I, respectively. ANOVA tables for Total Folates 1 and 2 are presented in Tables 17 - 18, respectively.

Table 17. Analysis of variance results for Total Folates 1

SOURCE	DF	TYPE I SS	F VALUE	PR > F
REP	1	15293.44	0.18	0.6791
COOK	2	282249.06	1.63	0.2243
STORE	1	33978.78	0.39	0.5388
COOK*STORE	2	372073.72	2.15	0.1465
TIME	2	3923065.39	22.72	0.0001*
COOK*TIME	4	5475888.78	15.86	0.0001*
STORE*TIME	2	330492.72	1.91	0.1780
COOK*STORE*TIME	4	1909640.78	5.53	0.0049*

Table 18. Analysis of variance results for Total Folates 2

SOURCE	DF	TYPE I SS	F VALUE	PR > F
REP	1	10226138.03	5.79	0.0278*
COOK	2	13686265.06	3.88	0.0410*
STORE	1	15130803.36	8.57	0.0094*
COOK*STORE	2	19100817.72	5.41	0.0152*
TIME	2	72882357.72	20.64	0.0001*
COOK*TIME	4	56089777.61	7.94	0.0008*
STORE*TIME	2	10874233.39	3.08	0.0722
COOK*STORE*TIME	4	59914432.28	8.48	0.0006*

The effect of cooking on Total Folates 1 and Total Folates 2 is graphically represented in Figures 19 & 20 while the changes in these variables during frozen storage are graphically depicted in Figures 21 & 22. As expected, total folate values were lower in cooked samples than in

raw samples. The percent reduction is presented in Table 19.

Table 19. % Reduction in total folates in raw and cooked samples.

	TOTAL FOLATE 1	TOTAL FOLATE 2
B1	55	32
B2	45	22
F1	55	36
F2	48	27

Total Folates 1 was significantly affected by length of storage and the interactions cooking by time and cooking by time by storage method. Total Folates 2 was a function of all treatments and interactions of treatments except for the interaction storage method by time.

Microbial assays. The raw data pertaining to the quantitation of liver folates by the L. casei assay are found in Appendix J. It should be noted that values reported on raw samples represent their total folate content. However, in cooked samples the extent of conjugase inactivation due to the different cooking treatments is unpredictable. Therefore, values from the L. casei assay of these samples might be smaller than their actual total folate content. Folate values by the

Fig. 19--Effect of cooking on Total Folates 1 (by HPLC, DHF not included).

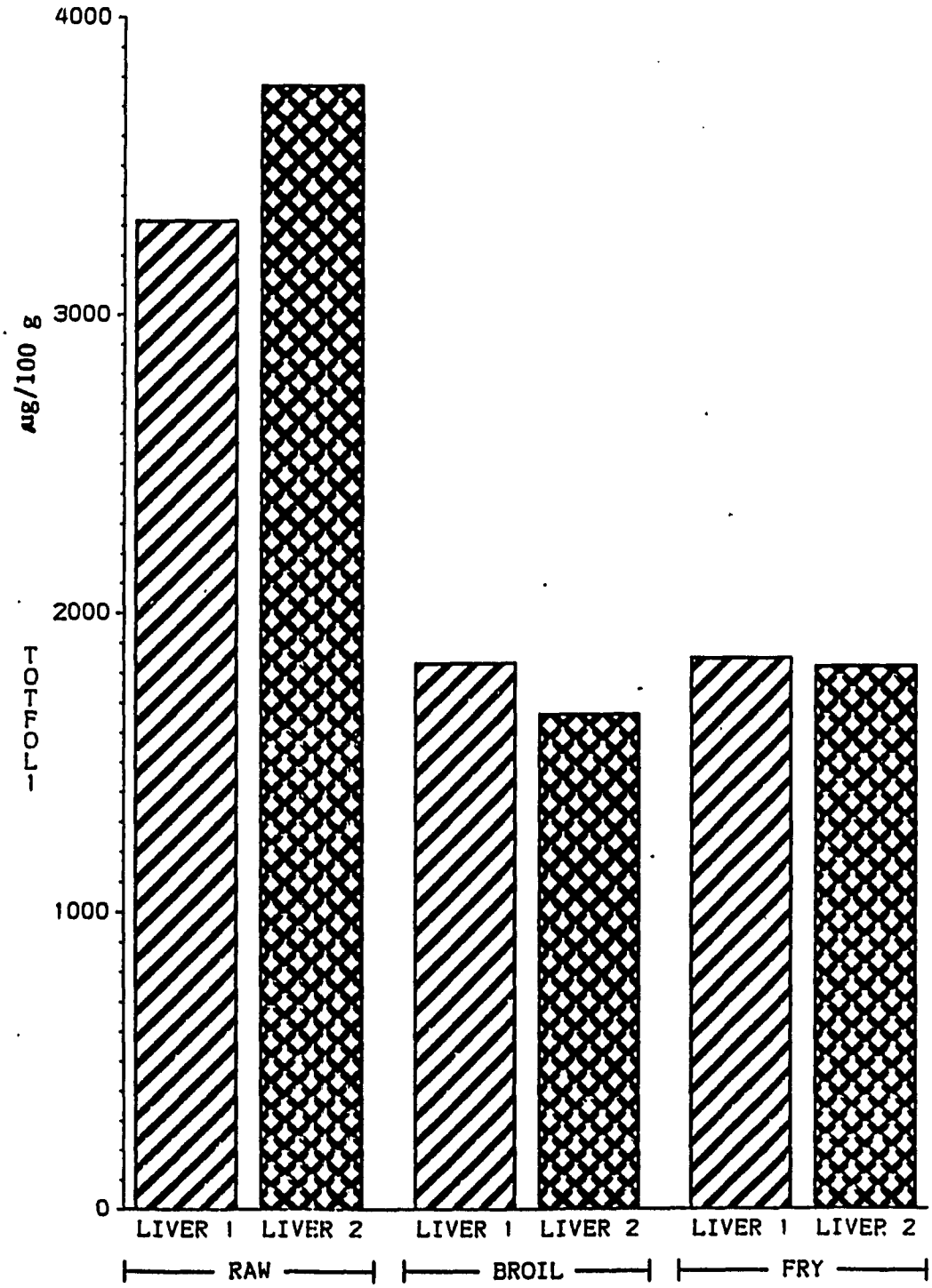


Fig. 20--Effect of cooking on Total Folates 2 (by HPLC, DHF included).

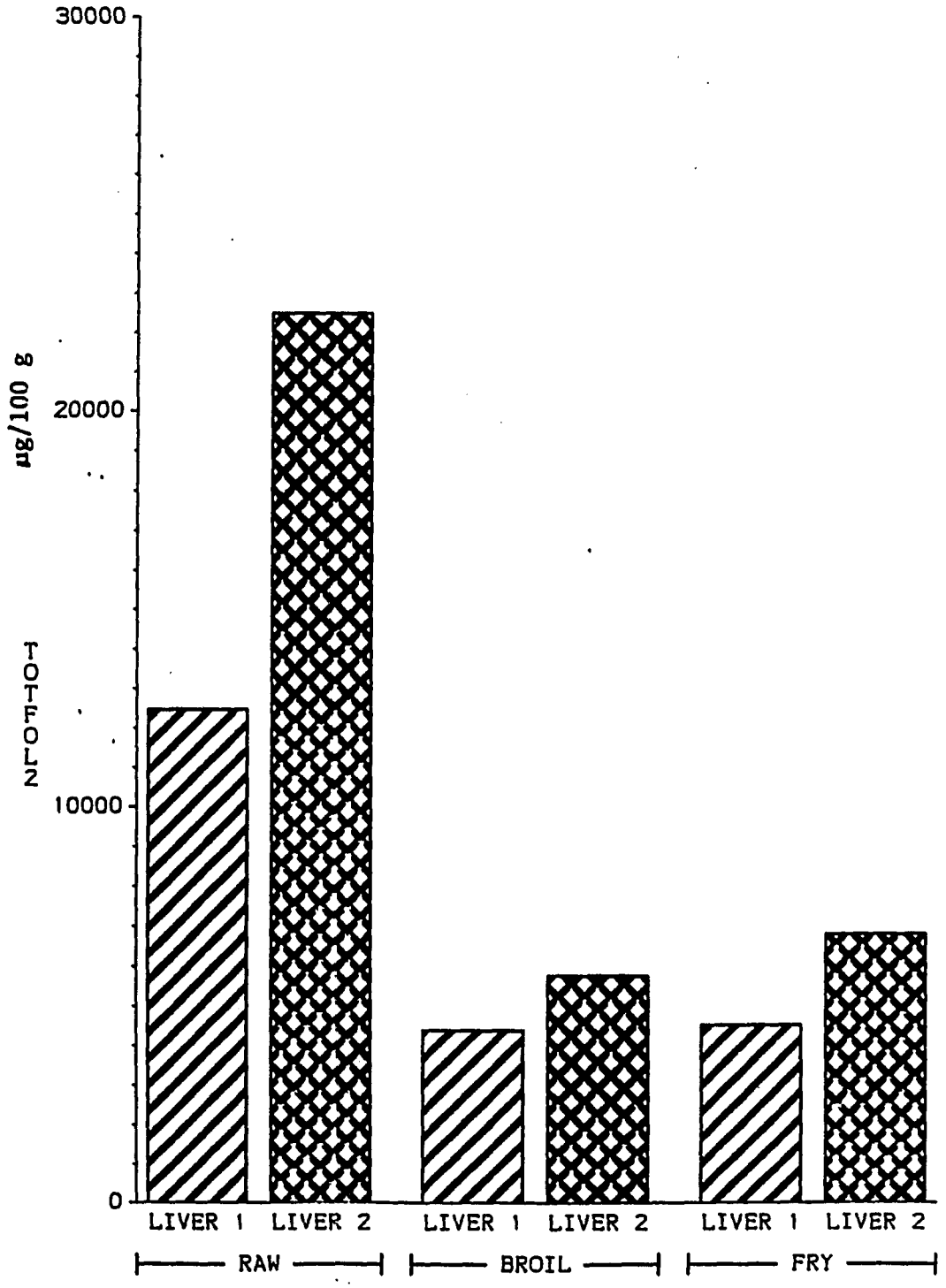


Fig. 21--Changes in Total Folates 1 (by HPLC, DHF not included) during frozen storage for different cooking and packaging treatments.

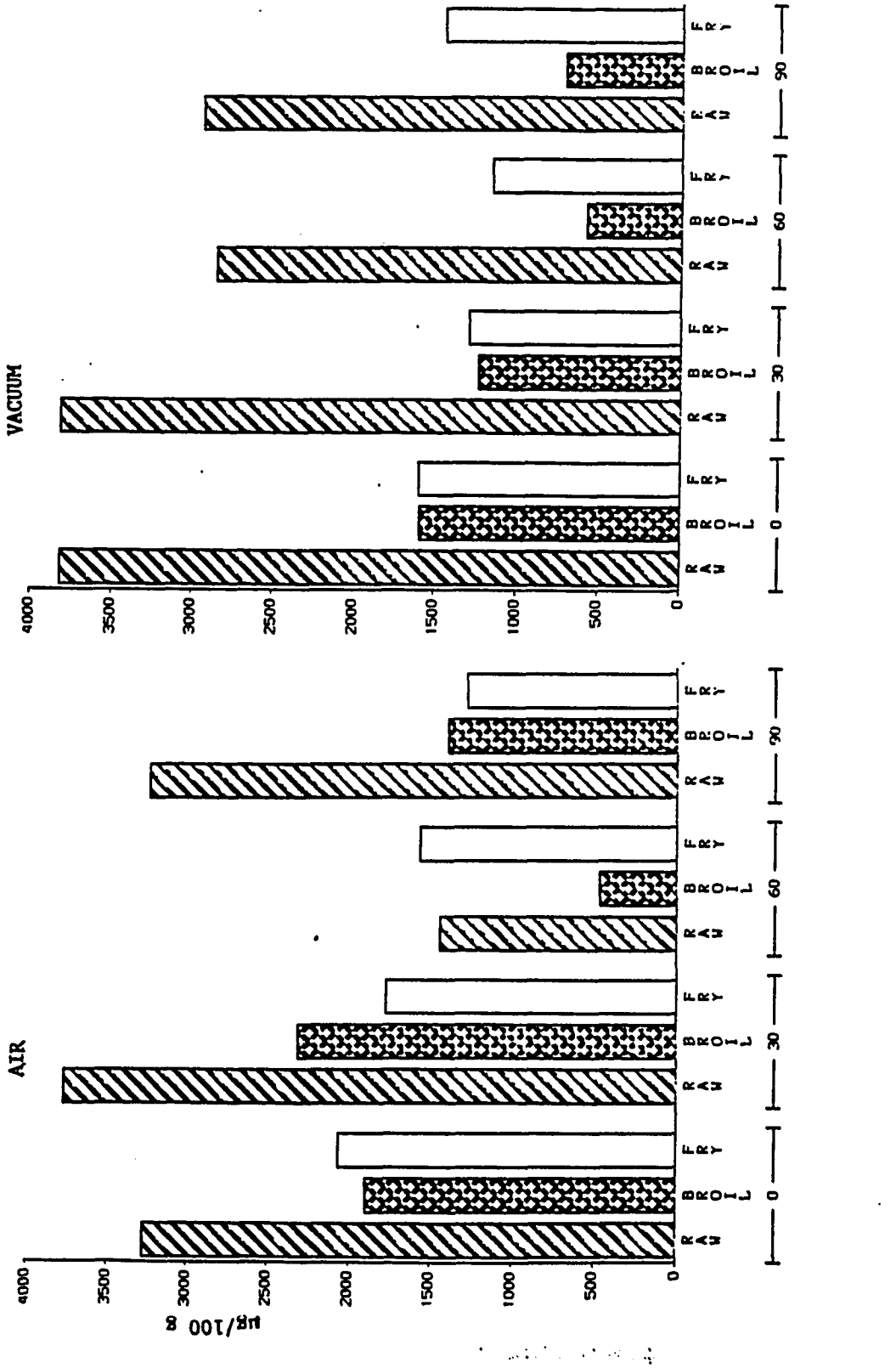
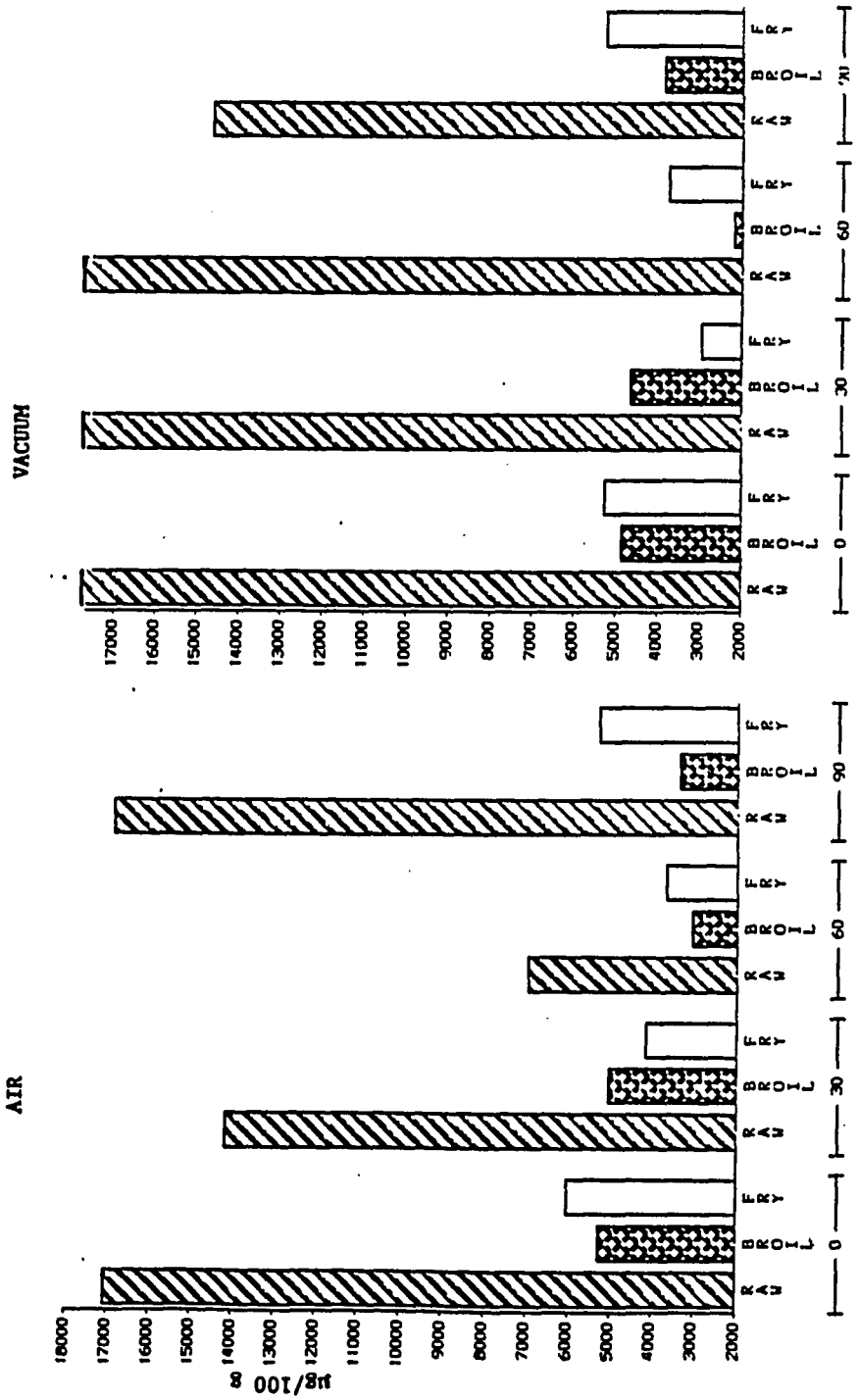


Fig. 22--Changes in Total Folates 2 (by HPLC, DHF included) during frozen storage for different cooking and packaging treatments.



microbial assays have been abbreviated "Total Folates 3". The ANOVA for this data is presented in Table 20.

Table 20. Analysis of variance for Total Folates 3

SOURCE	DF	TYPE I SS	F VALUE	PR > F
REP	1	24649.00	13.06	0.0021*
COOK	2	502608.17	133.11	0.0001*
STORE	1	317344.44	168.10	0.0001*
COOK*STORE	2	20597.06	5.46	0.0148*
TIME	2	173268.50	45.89	0.0001*
COOK*TIME	4	59380.83	7.86	0.0009*
STORE*TIME	2	7595.39	2.01	0.1644
COOK*STORE*TIME	4	86207.61	11.42	0.0001*

* = $p < 0.05$

The only non-significant factor appears to be the combination of storage method and time. The effect of cooking on Total Folates 3 is depicted in Figure 23. The change in this variable during frozen storage is represented in Figure 24. The L. casei activity exhibited by liver 1 and liver 2 reported as µg/100 g (dry basis), is presented in Table 21.

Table 21. L. casei activity of raw and cooked liver samples.

	Raw	Broiled	Fried
	µg/100g		
Liver 1	1003	590	482
Liver 2	1136	680	578

One interesting aspect of the folate values obtained by the microbial assays relates to the large difference found between these values and those obtained from the HPLC analysis. In fact, Total Folates 1 and Total Folates 2 tend to surpass Total Folates 3 by more than three fold and ten fold respectively. This can be seen in the following comparison of folate values in raw liver by HPLC analysis and microbial assays (Table 22).

Table 22. A comparison of folate values (µg/100g) in raw liver by HPLC and microbial assays.

	Folate Value		
	Total Folates 1	Total Folates 2	Folates 3
	ug/100g		
LIVER 1	3313	12453	1003
LIVER 2	3770	22461	1136

Total Folate 1 = THF + 5-CH_3-THF + 5-CHO-THF (HPLC)
Total Folate 2 = THF + 5-CH_3-THF + 5-CHO-THF + DHF (HPLC)
Total Folates 3 = *L. casei* activity

Fig. 23--Effect of cooking on Total Folates 3 (by microbial analysis).

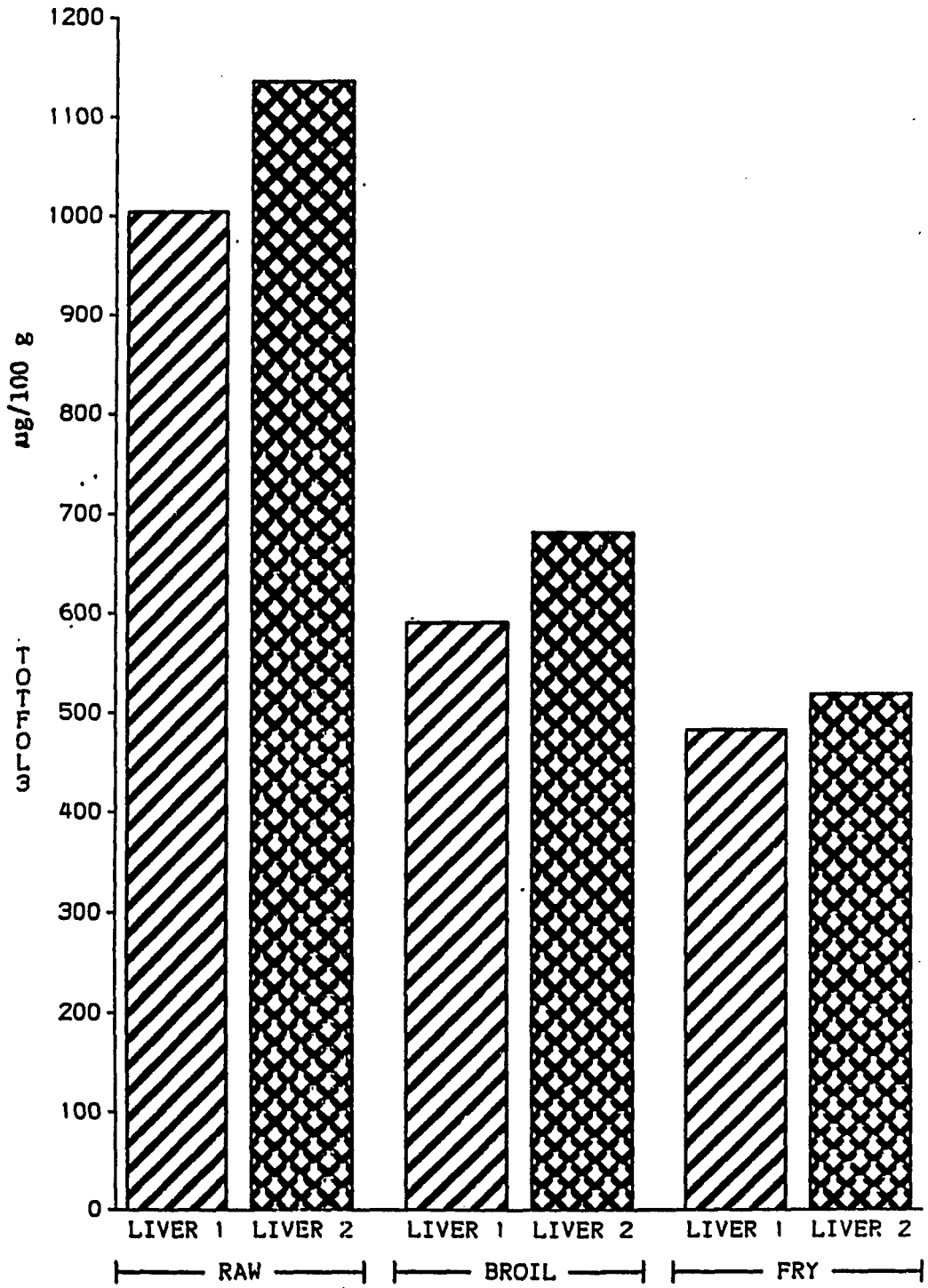
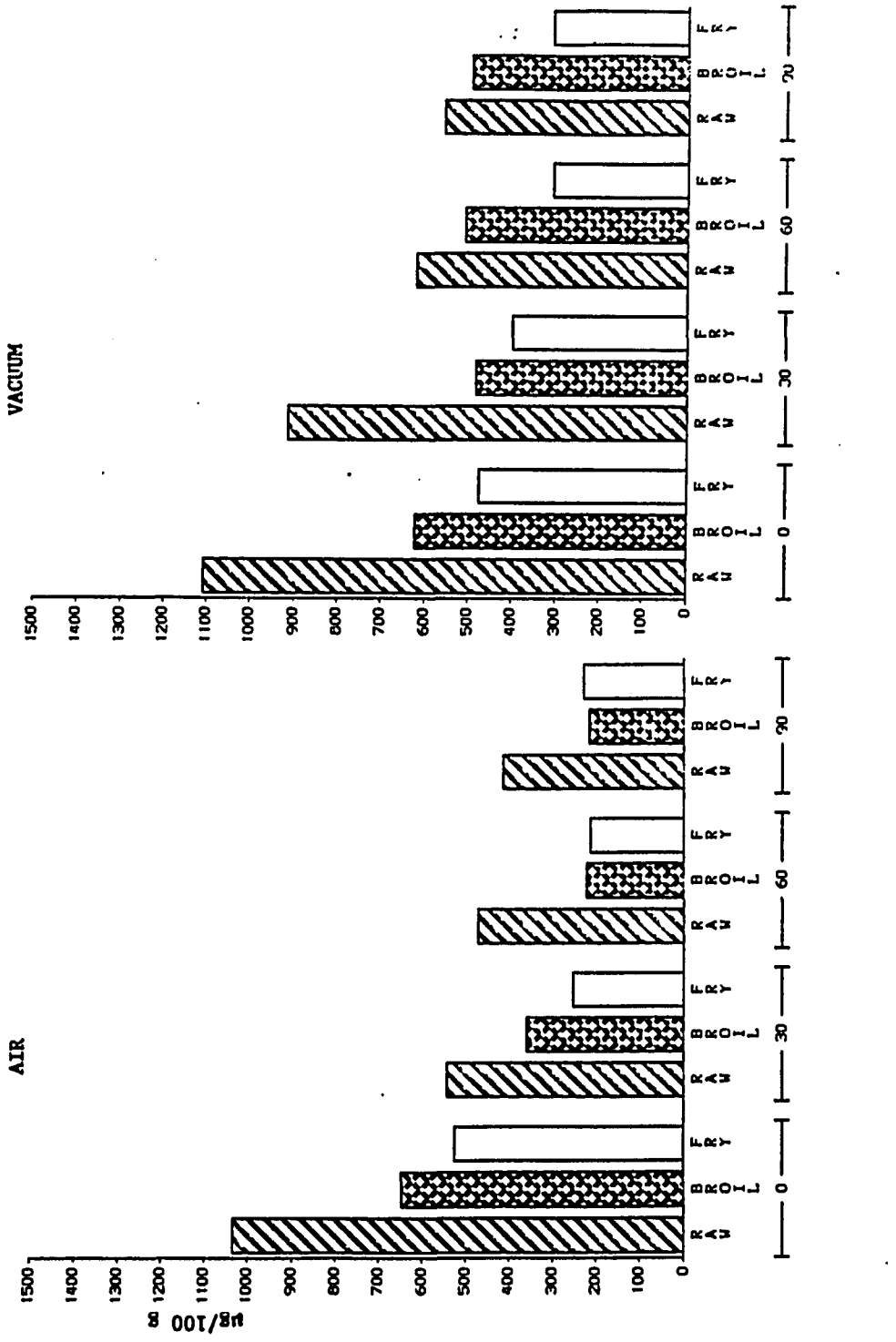


Fig. 24--Changes in Total Folates 3 (by microbial assay) during frozen storage for different cooking and packaging treatments.



Interactions. Interactions between the different treatments were analysed by SAS General Linear Model Procedure. The complete analysis can be found in Appendix K. It is an analysis of the change (difference from control) in any one variable due to a combination of cooking and storage treatments over time. These interactions are graphically represented in Figures 25-33. Statistically significant relationships are indicated in Table 24. These could be used as mathematical models capable of predicting the behavior of folates for specific cooking and storage conditions.

Correlations. Pearson Correlation Coefficients were established to indicate correlations between any of the folate derivatives by HPLC and the folate content as indicated by the L. casei assay. The intent was to relate the folate content of beef liver to its bioavailability. The letter "a" indicates a probability factor < 0.01 , while "b" indicates a factor < 0.05.

Fig. 25--Effect of treatment interactions on pt-6-COOH

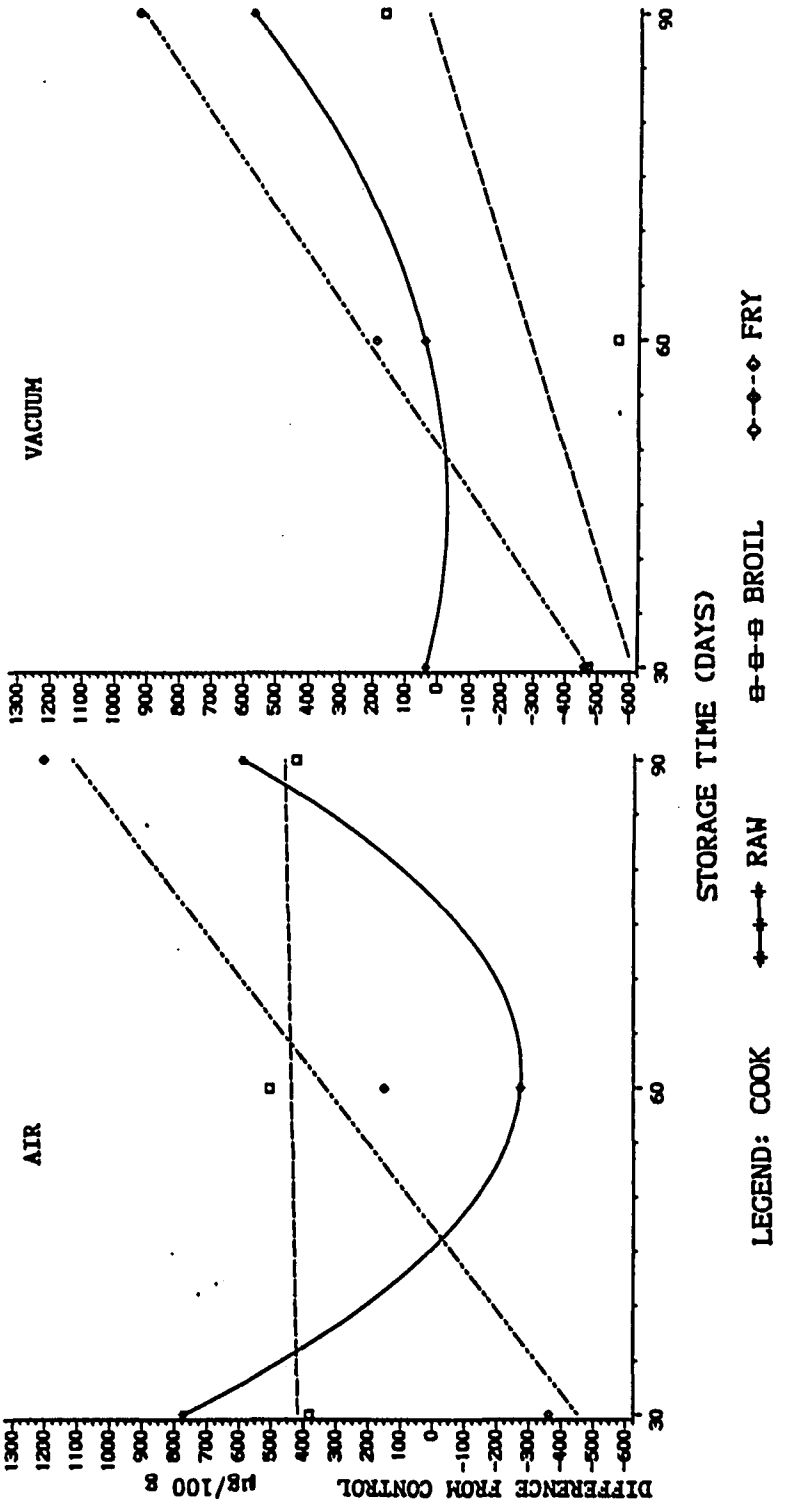


Fig. 26--Effect of treatment interactions on pABG

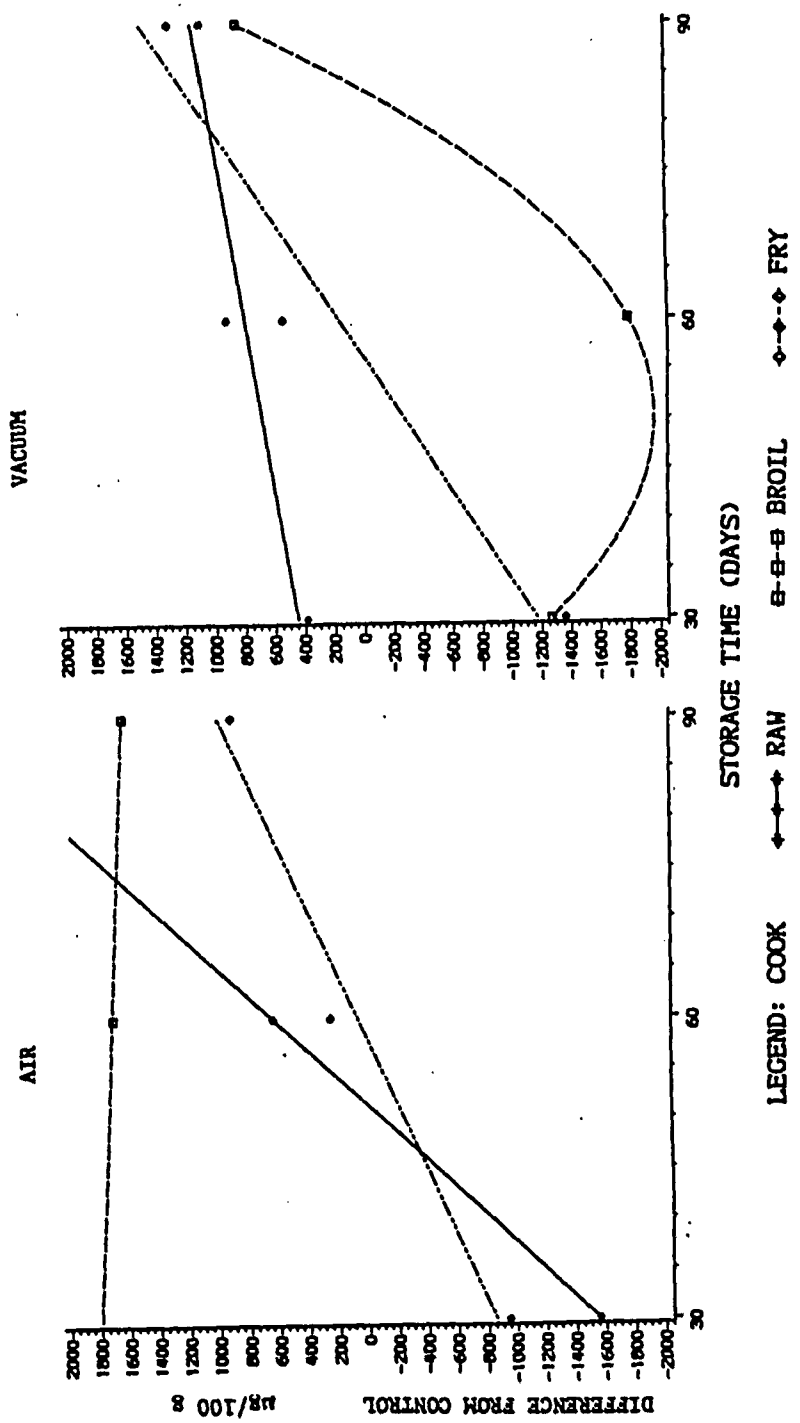


Fig. 27--Effect of treatment interactions on THF

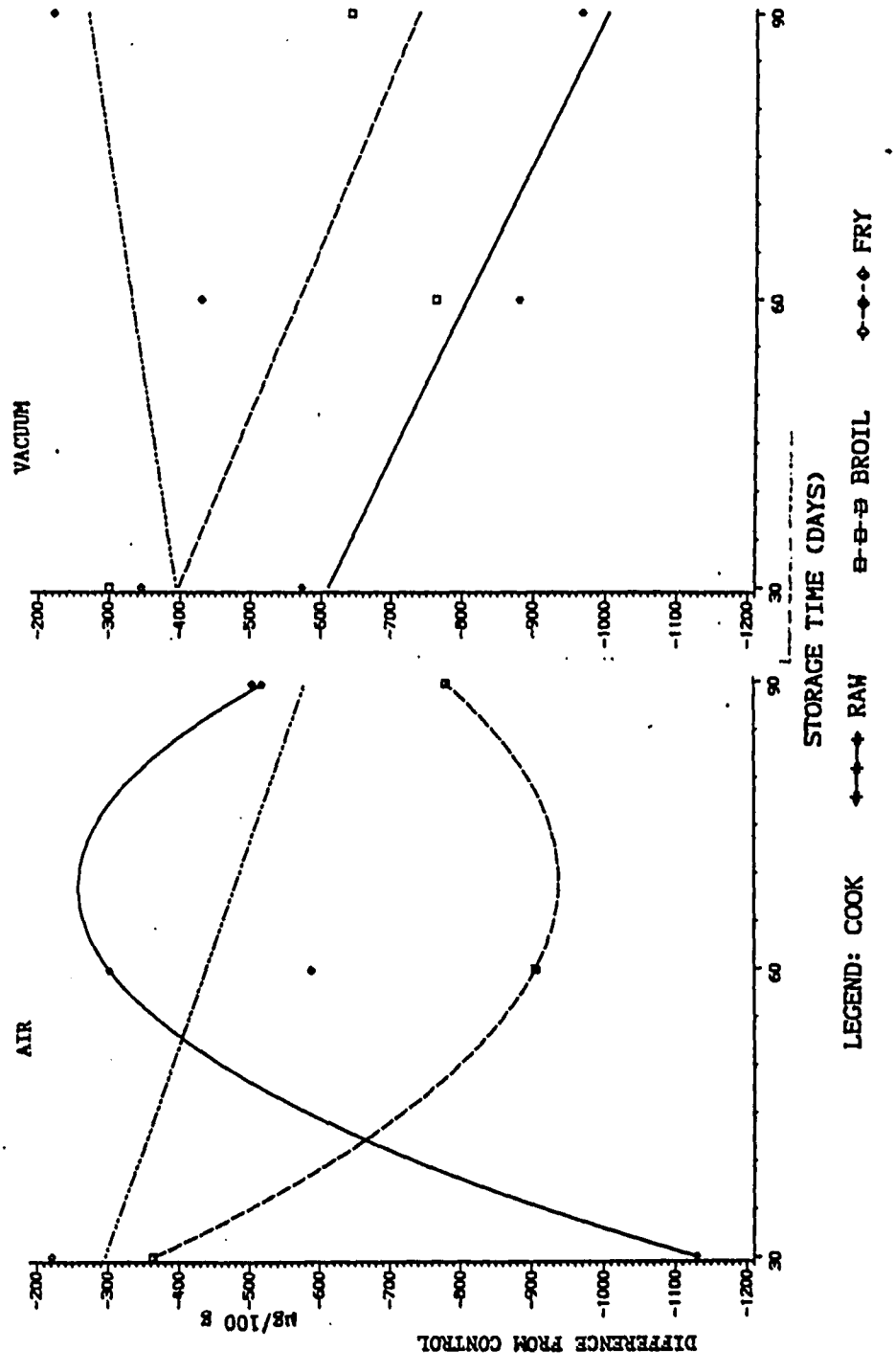


Fig. 28--Effect of treatment interactions on 5-CH_3-THF

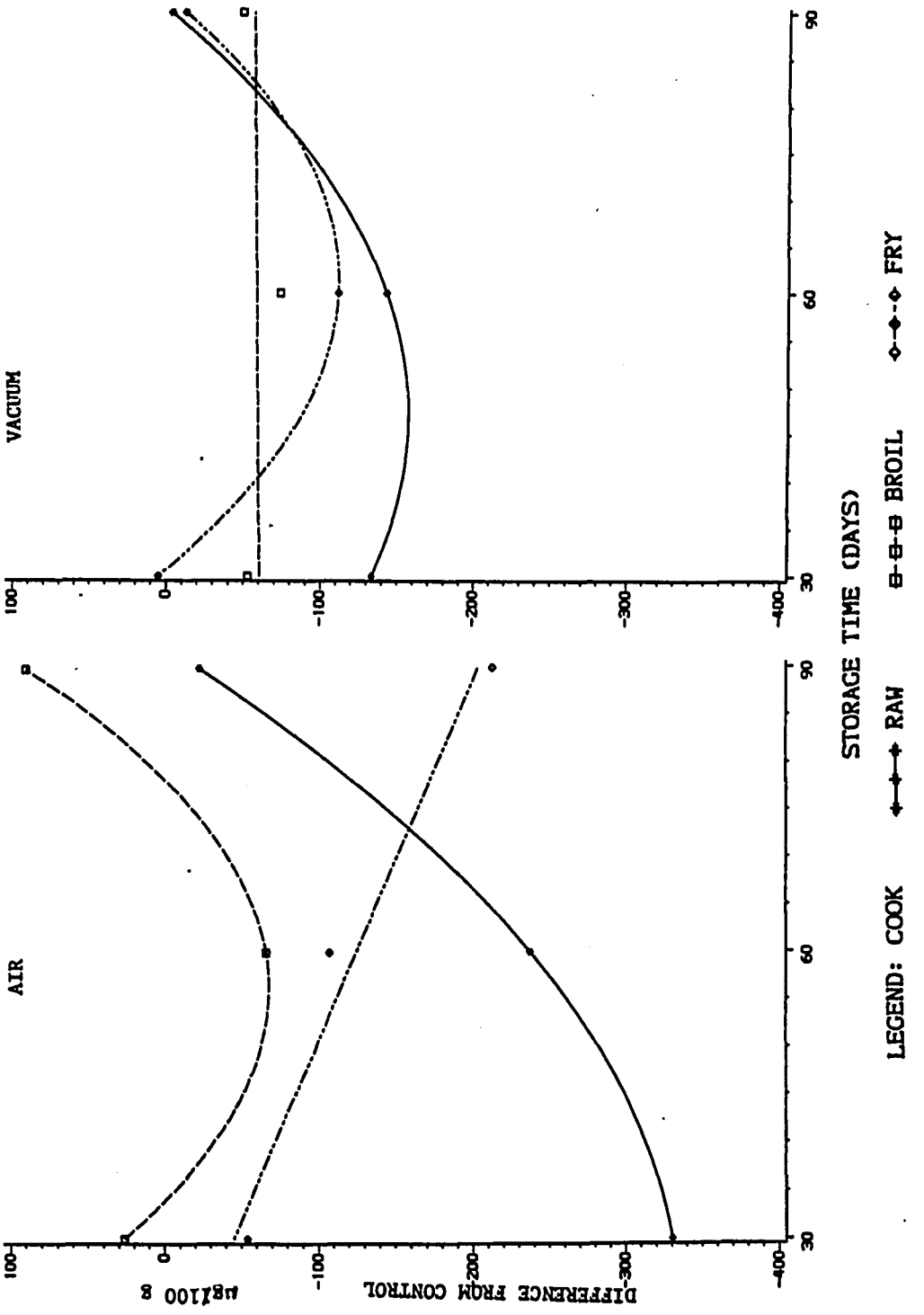


Fig. 29--Effect of treatment interactions on 5-CHO-THF

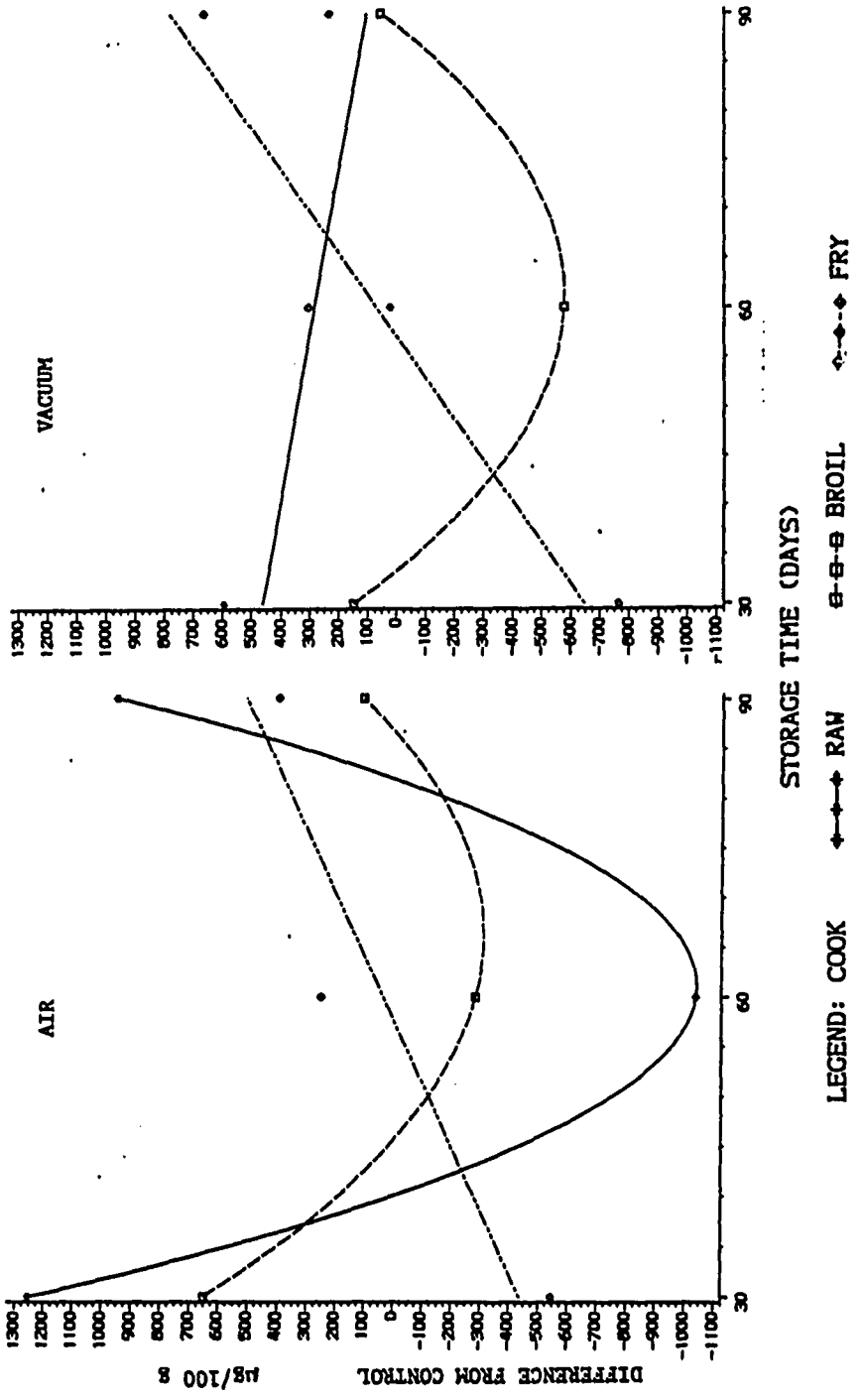


Fig. 30--Effect of treatment interactions on "DHF"

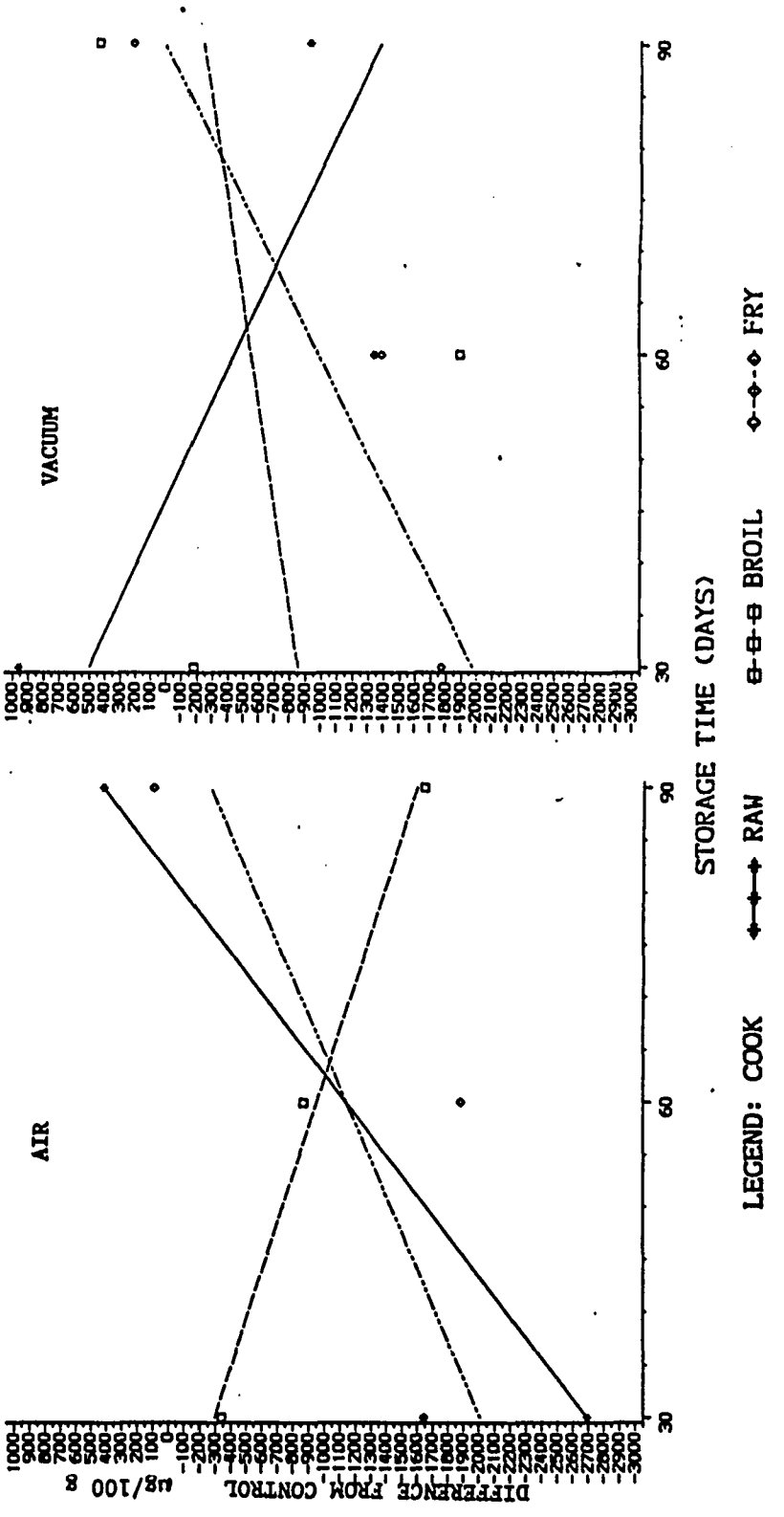


Fig. 31--Effect of treatment interactions on Total Folates 1 (by HPLC, DHF not included).

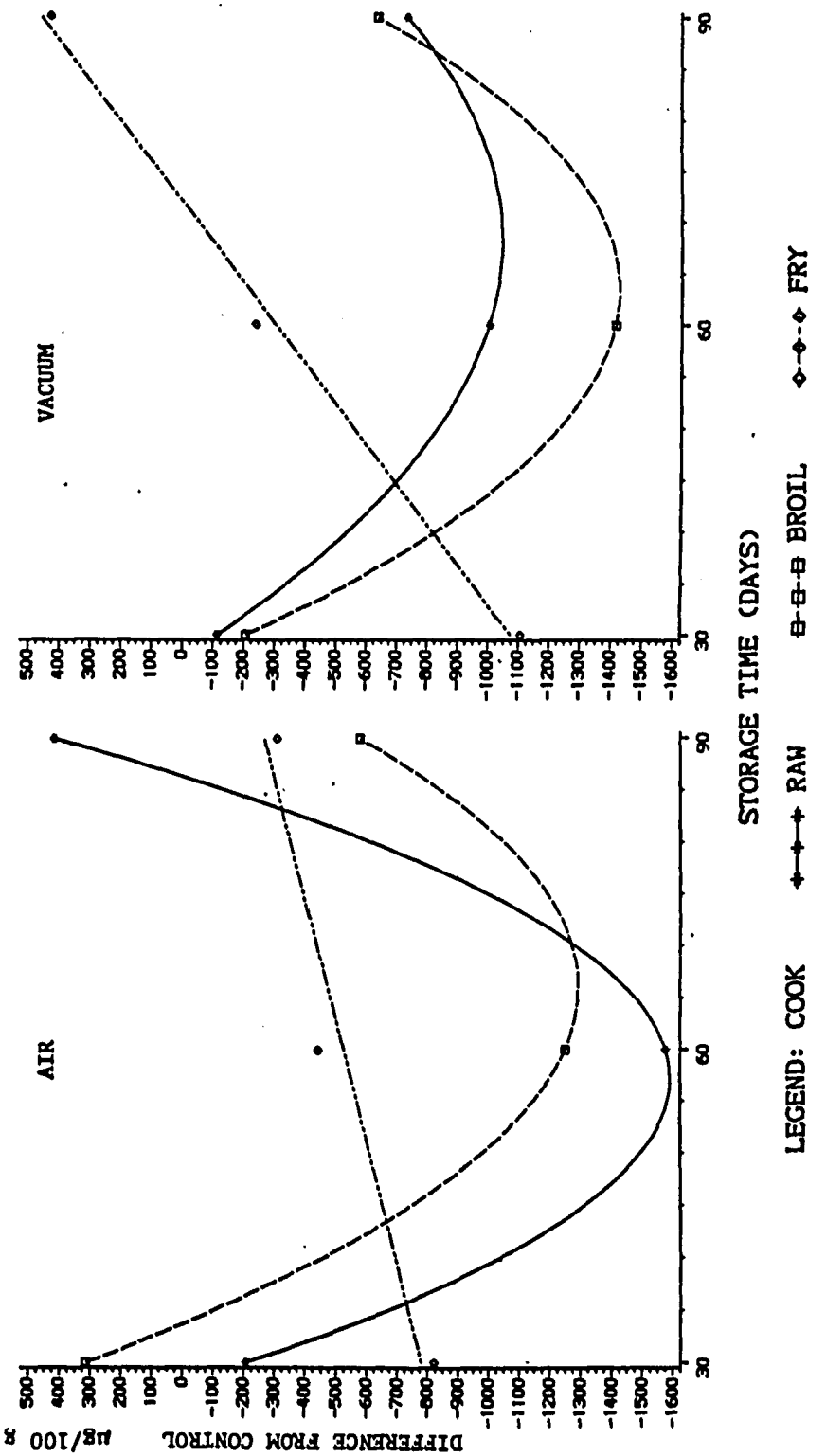


Fig. 32--Effect of treatment interactions on Total Folates 2 (by HPLC, DHF included).

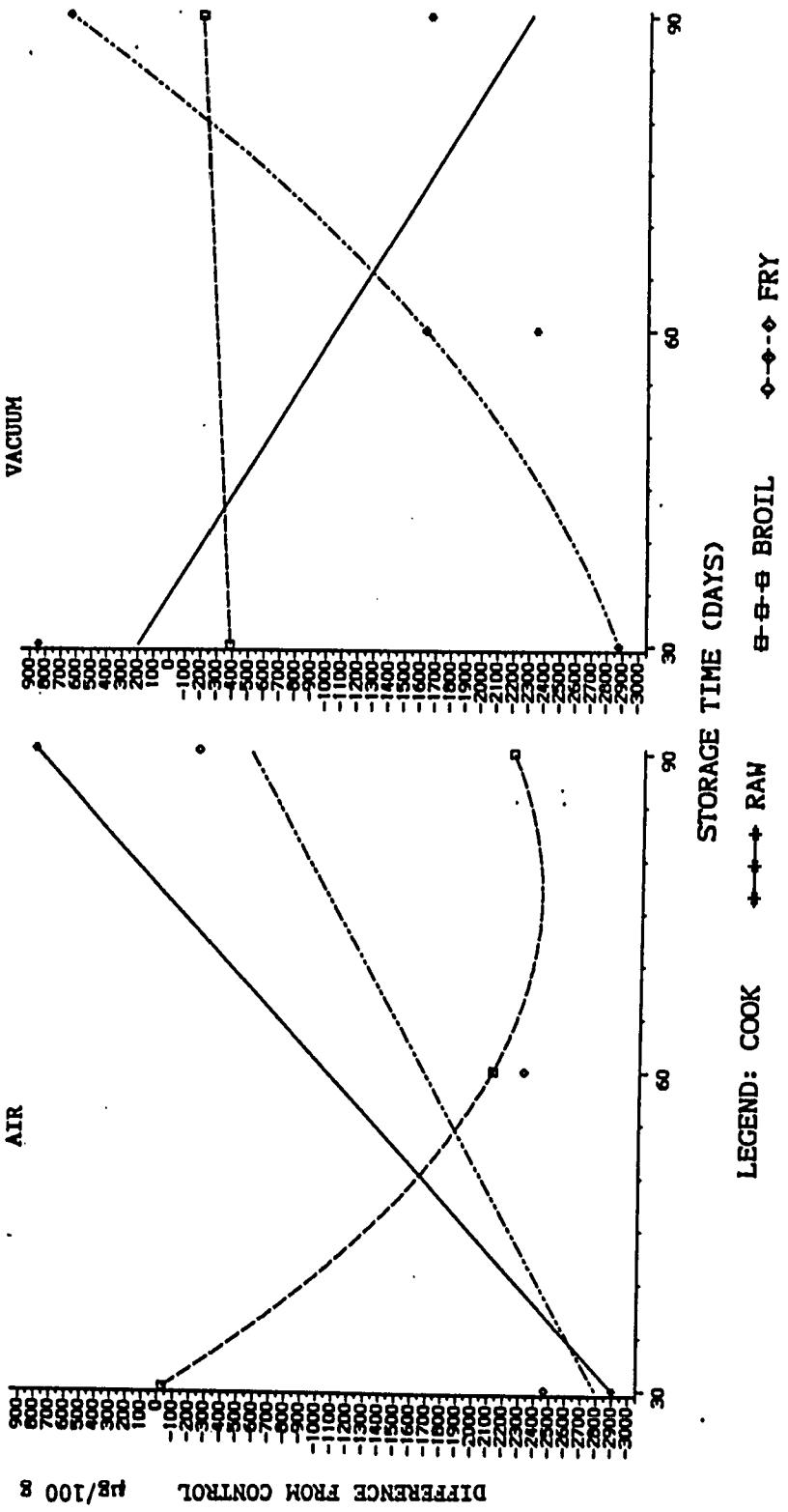


Fig. 33--Effect of treatment interactions on Total Folates 3 (by microbial assays).

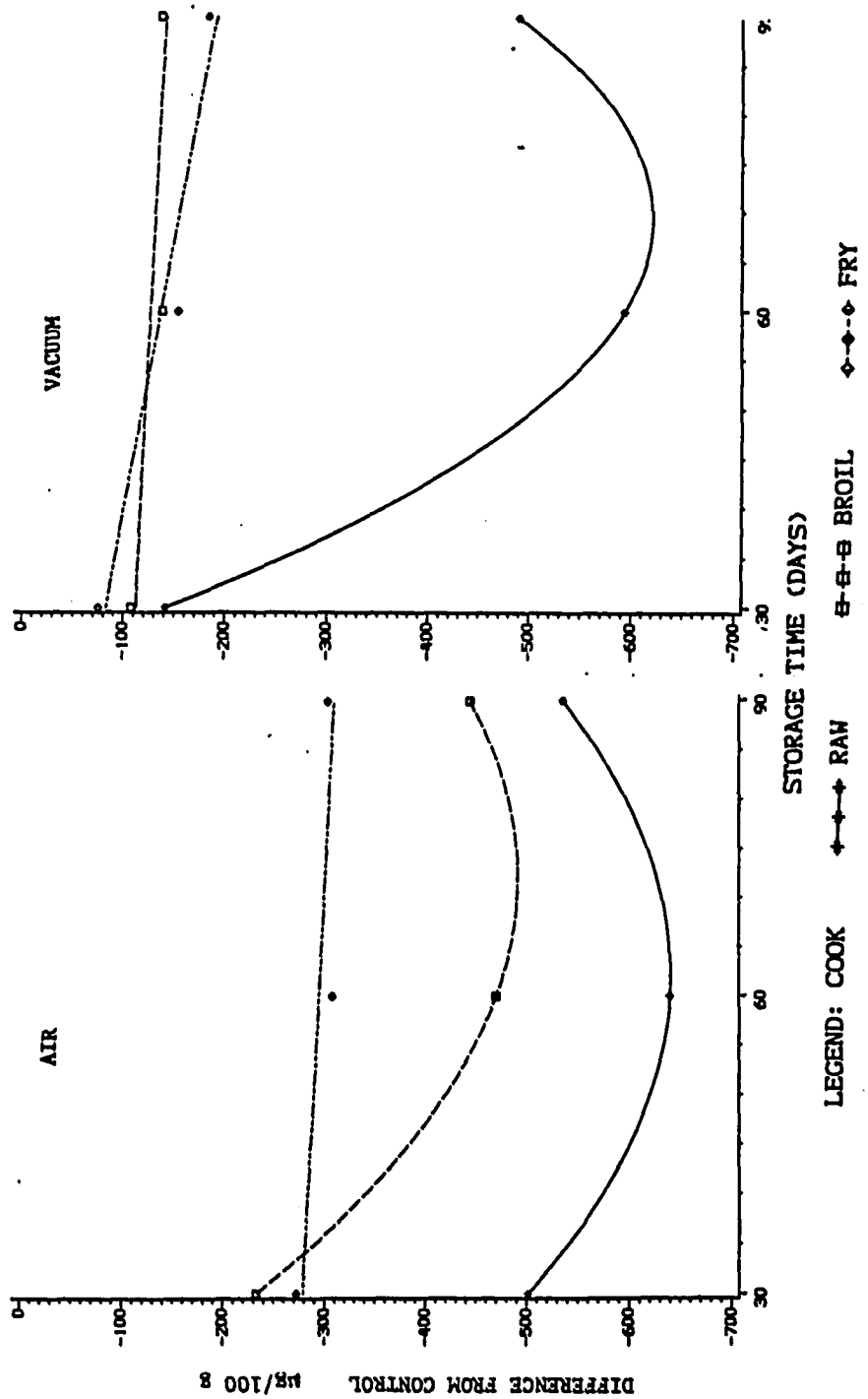


Table 23. Correlations by storage type

Air:	None significant	
Vacuum:	THF	0.785 a
	5-CH_3-THF	0.754 a
	DHF	0.758 a
	Total Folate 1	0.816 a
	Total Folate 2	0.780 a

Table 24. The interaction between cooking, storage type and freezing time.

	RAW				BROIL				FRY			
	AIR		VAC		AIR		VAC		AIR		VAC	
	L	Q	L	Q	L	Q	L	Q	L	Q	L	Q
pt-6-COOH												
p-ABG	a						a	a	a		a	
THF	a	a			b	b						
5-CH_3-THF	a	b	a	b		a			a			a
5-CHO-THF		a			b	a		a	a			a
DHF	b	a										
Tot. Fol.1	b	a						b			a	
Tot. Fol.2	b	a						b			b	
Folates 3		a	a	a	a	a					b	

L : Linear relationship
Q : Quadratic relationship

a : $p < 0.01$
b : $p < 0.05$

Table 25. Correlation coefficients by time of storage

30 days	5-CH_3-THF	0.887 a
	DHF	0.791 a
	Total Folate 2	0.798 a
60 days	5-CH_3-THF	0.843 a
	DHF	0.792 a
	Total Folate 1	0.830 a
	Total Folate 2	0.811 a
90 days	None significant	

Table 26. Correlation coefficients by cooking method

Raw	THF	0.841 a
Broil	None significant	
Fry	None significant	

Table 27. Correlation coefficients by cooking method and storage type.

Broil, Air	p-ABG	- 0.756
Broil, Vacuum	None significant	
Fry, Air	None significant	
Fry, Vac	None significant	
Raw, Air	THF	0.776 a
Raw, Vac	THF	0.921 a
	Total Folate 2	0.718 a

Table 28. Correlation coefficients by storage type and time.

Air, 30	5-CH_3-THF	0.856 a
	Total Folate 2	0.718 a
Air, 60	5-CH_3-THF	0.878 a
	DHF	0.854 a
	Total Folate 1	0.835 a
	Total Folate 2	0.882 a
Air, 90	THF	0.765 a
Vacuum, 30	5-CH_3-THF	0.916 a
	5-CHO-THF	0.752 a
	DHF	0.890 a
	Total Folate 1	0.872 a
	Total Folate 2	0.898 a
Vacuum, 60	THF	0.847 a
	5-CH_3-THF	0.884 a
	DHF	0.751 a
	Total Folate 1	0.859 a
	Total Folate 2	0.773 a
Vacuum, 90	THF	0.787 a

Table 29. Correlation coefficients by cooking method, storage type and time.

Broil, Air,	30	None significant		
	60	p-ABG	-0.981	b
		5-CH_3-THF	0.993	a
		Total Folate 2	0.996	a
	90	p-ABG	-0.984	b
		THF	0.975	b
		5-CHO-THF	-0.952	b
		Total Folate 1	0.950	b
Broil, Vacuum	30	p-ABG	0.975	b
		5-CH_3-THF	0.973	b
	60	p-ABG	0.977	b
		THF	0.968	b
	90	None significant		
Fry, Air,	30	Total Folate 2	0.996	b
	60	none significant		
	90	THF	0.969	b
		5-CHO-THF	-0.965	a
		Total Folate 1	0.950	a
Fry, Vac,	30	THF	0.985	b
		5-CH_3-THF	0.993	a
		5-CHO-THF	-0.993	a
	60	5-CH_3-THF	0.994	a
		Total Folate 1	0.993	a
		Total Folate 2	0.956	a
	90	None significant		
Raw, Vac,	30	THF	0.975	b
	60	THF	0.992	a
	90	p-ABG	-0.949	b

Table 30. Overall correlation coefficients

	r	p
pt-6-COOH	0.260	0.0270
p-ABG	-0.386	0.0008
THF	0.654	0.0001
5-CH_3-THF	0.695	0.0001
5-CHO-THF	0.454	0.0001
DHF	0.708	0.0001
Total Folate 1	0.699	0.0001
Total Folate 2	0.729	0.0001

If these correlations are to be used as possible indicators of the L. casei activity of the samples, the following general trends could be established. No good index existed for non-vacuum storage while THF and Total Folates 1 constituted fairly good indices for storage under vacuum. As for freezing time, 5-CH_3-THF could be regarded as a good indicator of folate availability after storage for 30 and 60 days but not after 90 days. Finally, no good index of L. casei activity was established for different cooking treatments except for THF in raw liver.

When a combination of treatments was considered, highly significant correlations were generally established for cooked samples with p-ABG and 5-CHO-THF indicating negative relationships.

Although the overall correlation coefficients were statistically significant for all factors under consideration, none of the r values were greater than 0.75. Therefore, no dependable correlation was established.

CHAPTER IV

DISCUSSION

Evaluation of the Extraction Procedure

The extraction procedure was an adaptation of the method used by Day and Gregory (1981). Preliminary experiments, in which the pH, incubation time and percent ascorbate were varied, revealed different optimum conditions for the extraction of liver folates. Accordingly, the concentration of ascorbate in the homogenizing solution was increased from 0.25% to 2.0% and the final pH was raised to 4.5. At this pH, conjugase activity would be at its optimum level (Silink et al., 1975).

A comparison to the data reported by Day and Gregory (1981) of the different folate derivatives shows that the extraction procedure used during this study was superior (Table 31).

The use of ascorbate has been shown to protect reduced derivatives of folic acid (O'Broin et al., 1975) and has been advocated for such a function during extraction, chromatography and assay procedures of folates. Day and Gregory (1983) reported that the use of ascorbate in liquid model food systems reduced the oxygen partial pressure within the models thus enhancing the stability of folic acid and 5-CH_3-THF.

Table 31. Comparison[1] of percent recoveries for different folate derivatives.

Product	Day & Gregory Study	Present Study
pt-6-COOH	NR[2]	87
p-ABG	NR	90
THF	65	72
5-CH_3-THF	82	82
5-CHO-THF	73	81
DHF	NR	87
FA	NR	89

1 - Comparison done on raw liver samples
2 - NR=Not Reported

On the other hand, Wilson and Horne (1983) used HPLC analysis and L. casei assays to evaluate the protective effect of ascorbic acid on folate derivatives. Their results indicated that upon heating at 100°C for 10 min, folate solutions in 2% sodium ascorbate underwent significant interconversions. These interconversions were believed to be due to the formation of formaldehyde from ascorbate. However, it is unlikely that such interconversions took place in the present study since liver homogenates were heated in a water bath set at 95°C for only 5 min under a saturated nitrogen environment. This conviction is supported by the fact that pteroylglutamic acid did not appear in any of the samples, although Wilson and Horne (1983) determined that it was a product of DHF conversion. Therefore, the use of 2%

ascorbate during extraction of folates from food sources might be required for proper quantitation of folates. At this time, it is considered acceptable in the absence of severe heat treatment. Nitrogen saturation of the food homogenates and the complete absence of artificial illumination during the whole extraction procedure are strongly recommended, also.

However, it should be noted that an adequate heat treatment is essential. With insufficient heat treatment, several enzymes responsible for folate interconversions could remain active. A study by Bird et al. (1968) showed that these enzymes were not completely inactivated by heating at 75°C for 30 min. Rapid heating of small liver slices at 95°C in ascorbate buffer inhibited enzymatic activity and provided optimum conditions for obtaining unchanged folate derivatives. Therefore, the use of a 5 min heating at 95°C, as was applied in this study, is recommended for the inactivation of endogenous enzymes during extraction of folates from biological material.

The Folate Profile of Raw Bovine Liver

The folate derivatives of raw beef liver were reported by Day and Gregory (1981) to be mainly THF, 5-CH_3-THF and 5-CHO- and/or 10-CHO-THF. There was no attempt to identify and quantitate 10-CHO-THF in this study since it is expected to undergo rapid conversion to the 5-formyl derivative during the extraction procedure (O'Brion et al.,

1975; Silverman et al., 1961). A comparison of the folate composition of beef liver in this study with the Day and Gregory study is found in Table 32.

The absence of pteroylglutamic acid in liver samples is not unusual, since natural folates occur mostly as reduced derivatives of folic acid (Butterworth, 1968). More specifically, pteroylglutamic acid has not been reported in liver tissues before except in cases where a pharmaceutical preparation had been administered. A peak eluting at the same retention time as DHF was constantly detected in all liver extracts.

Table 32. The folate profile of raw beef liver

Study	Derivative (% of total)			
	THF	5-CH_3-THF	5-CHO-THF	DHF
Day and Gregory	23	22	55	--
Present				
Liver I[a]	41	12	47	--
Liver I[b]	11	3	12	74
Liver II[a]	36	13	51	--
Liver II[b]	6	2	9	83

a - DHF unaccounted for
b - DHF included

A high DHF content of liver has not been previously reported in the literature. Any folate or dihydrofolate present in dietary material is actually believed to

undergo rapid enzymatic reduction to tetrahydrofolate through the reaction:

$$NADPH + H^+ + DHF \overset{DHFR}{\underset{<----}{----->} } THF + NADP^+$$

(Blakely and Benkovic, 1984; p. 194)

The enzyme, dihydrofolate reductase (DHFR) that catalyzes this reaction has been the subject of thorough investigation. The Michaelis constant for bovine liver DHFR was reported as 6uM for DHF and 15uM for NADPH (Lai et al., 1982). However, it should be noted that these values are dependent on the specific ionic strength and pH of the assay medium. Moreover, in the case of DHF, these values have been determined at finite NADPH concentrations rather than by extrapolation to saturated NADPH. Therefore, the rate at which DHF is enzymatically reduced to THF might not be as high as it is thought to be. The result could be that material may have higher DHF concentrations than previously reported. The reason that DHF has not been fully accounted for might be due to its extreme lability which would incur large losses during the folate extraction procedures currently applied.

O'Brion et al. (1975) reported that DHF was stable for several days when stored as a suspension in 0.005M HCl at 4°C. However, when solutions of higher pH values were used in the absence of antioxidants, the rate of DHF degradation could not be measured due to the rapid loss of its biological activity (half-life <10 min). Similar findings

were reported by Brown et al. (1973) and Maruyama et al. (1978), who suggested that DHF was relatively stable under acidic conditions but rapidly degraded in basic solutions. Since the extraction of folates from biological material is usually performed in a pH 6.1 buffer, a high DHF content of bovine liver, if confirmed, in this study might be due to the use of a pH 4.5 homogenizing buffer and to other precautions taken during the extraction procedure. These precautions included the use of a protective 2% ascorbate, nitrogen saturation during most of the process and rapid freezing of the extracts under nitrogen at -80°C. This hypothesis is supported by the fact that DHF content of raw liver maintained at -20°C in non-vacuum packages decreased sharply after 60 days, while that of vacuum packaged samples remained basically unchanged, proving that DHF is subject to great oxidation under unfavorable conditions.

Also, it may be argued that the high DHF levels were the result of the THF oxidation. Although this possibility exists, it should be noted that the use of ascorbate has been shown to stabilize solutions of THF (Scrimgeour, 1980) and that such reactions usually occur under strong oxidizing conditions at a pH of 9.0 or 10.0 (Blakley and Beukovic, 1984; p. 184). The conditions of the extraction procedure may have been such that the enzymatic reaction of DHF synthesis was activated while that of DHF reduction was inhibited. Thus the enzyme thymidylate synthase catalyzing

the reaction:

$$dUMP + 5,10\text{-}CH_2\text{-}THF \text{ -----> } dTMP + DHF$$

could have been subjected to activation by any of the ions present in solution while DHFR would have been inhibited by the same ion, or by any other potential inhibitor.

This discussion suggests several possible explanations for a high DHF content of the liver samples in this study. However, this is the first report of high levels of DHF in biological tissue. Future research should be directed towards clarifying this finding. Analytical techniques, such as Nuclear Magnetic Resonance (NMR) (Poe, 1980) or electron impact Mass Spectrometry (Smith et al., 1981), could be employed in this regard. These results suggest a need to re-evaluate the current methodologies used for folate extraction from foods as well as existing dietary folate tables.

The Effect of Cooking on Liver Folates

With the exception of THF, the folates monitored in this study were destroyed by broiling to a greater extent than by frying. This result may be a reflection of the fact that broiled liver reached an internal temperature of 70°C more slowly than those which were fried (20 and 11 min, respectively). Thus, during broiling the livers were exposed to the heat source for a greater period of time. The thermal stability of the folacin derivatives was determined to be:

(DHF) < 5-CHO-THF < 5-CH_3-THF < THF

These results were different from the previously reported data on the thermal stability of folates (Cooper et al., 1978). This study indicated that the order of stability in microwave and conventional heating was:

THF < 5-CH_3-THF < 5-CHO-THF.

Their study was performed on standard folate solutions by heating to 100°C over a heating mantle. Moreover, the folate activity after each heat treatment was measured by the L. casei assay. The limitation of such methodology is the impracticality of projecting the behavior of a nutrient in a water solution to a complex biological matrix such as liver. The superiority of HPLC analysis as a direct measure of folate levels over an estimation by microbial analysis has been demonstrated (Chen and Cooper, 1979). A study in the same laboratory evaluated the stability of THF and 5-CH_3-THF under various conditions. In this study, the authors observed a very erratic behavior of THF at temperatures below 100°C. They concluded that temperature effects alone could not account for their data. However, they noted that the thermal destruction of folacin is mainly an oxidative process, since the stability of THF increased in the presence of ascorbate and nitrogen atmosphere. The presence of natural antioxidants in beef liver which could provide a protective effect on THF is a possibility. Antioxidants, which are reducing agents,

would have an opposite effect on DHF. Since the product of DHF reduction is THF (Wilson and Horne, 1983), the thermal stability observed for THF might be an artifact of DHF reduction.

The greater thermal stability of the methyl versus the formyl derivative upon cooking has not been previously reported. The heat destruction of 5-CH_3-THF was observed to follow first order kinetics (Chen and Cooper, 1979), while other studies suggested that this folacin derivative has great thermal stability in liquid model food systems (Day and Gregory, 1983).

With regard to the oxidation products, an increase in p-ABG levels was expected since it is the resultant product of the C9-N10 bond cleavage of folates along with pt-6-COOH (and other pterins). Although no data could be found in the literature on the thermal stability of p-ABG and pt-6-COOH, the results of this study suggest that pt-6-COOH might be very heat labile, being continuously destroyed during cooking as it is being produced from the breakdown of folates.

This study has produced for the first time a profile of the thermal stability of the major folate derivatives in an actual food system. The fact that the findings do not agree with previous work performed on folate solutions should constitute an incentive to direct any future research on the thermal stability of folates towards actual food systems.

Effect of Storage on Liver Folates

The behavior of individual folates and oxidation products upon storage seems erratic and inconsistent. The changes in each of the variables cannot be solely attributed to its stability profile under given storage conditions. Dynamic interconversions cannot be ruled out since the extent of enzyme inactivation due to the different cooking treatments is unknown. Moreover, the reappearance of certain folates at elevated levels after 90 days of storage could be due to the slow deconjugation of pteroylpolyglutamates or to microbial synthesis by contaminating organisms. Other important factors contributing to the erratic results could be the liberation of folacin from proteinaceous folate binders and the high localization of liver folates. Localization of folates has been previously reported in sheep liver (Gawthorne and Smith, 1973), indicating that such intracellular compartmentalization is to be expected in beef liver too.

The loss of THF in non-vacuum packaged raw samples was greatest after the first month of storage. However, when stored under vacuum, only about half was lost. Therefore, THF appears to be lost both to oxidative and enzymatic processes. After 60 days of storage in non-vacuum packages, THF levels rose again in raw liver, but decreased in broiled and fried liver. An explanation for this could be that most of the oxygen is consumed during the first 30 days of storage and THF is then regenerated from DHF by the action of DHFR. The sharp decrease in DHF levels between 30 and 60 days (only 37% remained after 60 days compared to 79% after 30 days) supports this theory. Therefore, the fact that THF levels decreased in broiled and fried liver could be an indication of DHFR inactivation due to cooking. In vacuum packaged samples, the decrease in THF levels after 30 days of frozen storage is almost the same irrespective of the cooking treatment. This might be due in great part to the oxidative effect of the dissolved oxygen left in the livers. The rise in the 5-CHO-THF content of raw and broiled livers after 30 days of storage could, therefore, be attributed to the conversion of 5-CH_3-THF rather than THF.

The loss of THF during storage in either vacuum or non-vacuum packages appears to be due primarily to oxidative rather than enzymatic processes. In raw liver stored under aerobic conditions, a regeneration of THF from

DHF seems to take place after 60 days. No similar trend occurs under anaerobic conditions. This might be due to an inactivation of DHFR caused by a drop in pH or to a depletion of NADPH required for the reaction.

The greatest losses in 5-CH_3-THF occurred in raw liver stored in non-vacuum packages. However, the levels of this folate vitamer steadily increased with storage time. This finding was again probably due to enzymatic interconversions. A similar pattern was seen in vacuum packaged raw liver although the initial loss was much smaller. Under the same storage conditions, broiled liver had similar initial losses which were not accentuated by longer storage periods. Therefore, a mechanism similar to the one described for THF could be taking place. Thereby, oxidative losses which occur during the first 30 days consume all of the oxygen initially present. Additional change would not be observed, since the heat generated during broiling would stabilize further enzymatic activity. Muryama et al. (1978) reported the product of 5-CH_3-THF oxidation as 5-CH_3-DHF. The latter was not included in HPLC analysis of folates. This may account for some erratic results concerning 5-CH_3-THF.

Finally, the loss of the oxidation product p-ABG at 30 days of storage in raw and fried samples non-vacuum packaged could be explained by previous reports on the susceptibility of p-ABG to oxidation (Muryama et al., 1978).

Total Folates

The most striking feature of the total folate values was the large discrepancy between the values obtained by the HPLC analysis and those derived from the L. casei assay. One main factor that accounts for this discrepancy could be related to the possible high DHF content of the liver samples. O'Brion et al. (1975) indicated that they were unable to study the nutritional stability of this compound by the L. casei assay due to its extreme lability even in the presence of 0.6% ascorbate under nitrogen. Since DHF would have been destroyed during the microbial assay it would have been unaccounted for. However, even under these circumstances, the total folate values obtained by HPLC, with the exclusion of DHF (Total Folates I), remain about three times higher than those determined by the microbial assay. This could be partly due to the variability in the response of L. casei to the different folate derivatives (Phillips and Wright, 1981). The occurrence of folate forms in liver that are not utilizable by L. casei is to be considered, also. Ristow et al. (1982b) reported higher values for liver folates when chick

bioassays were used as compared to the L. casei assays. Another possible factor is the relative ability of the two methods to differentiate between folates of various glutamate chain lengths. Since an exogenous source of conjugase was not employed, total folate determinations for both cooking treatments may have been adversely affected by an inactivation of the endogenous conjugase. However, previously published research indicates that bovine γ-glutamyl hydrolase is a heat resistant enzyme that suffers very little thermal denaturation even after 30 min of heating at 65°C (Silink et al., 1975). The enzyme would probably have greater stability in an actual food system due to the protective effect of proteins. A study on chicken liver conjugases reported irreversible inactivation of the enzyme after heating at 100°C for 15 min. It is very improbable that total inactivation took place in this study since the cooking treatment was terminated the moment the internal temperature of the samples reached 70°C. Subsequently, they were cooled rapidly on ice. This fact can be further supported by the observation that values for Total Folates I by HPLC were about three fold higher than those of Total Folates by microbial assay irrespective of the cooking treatment.

Liver folates have been reported to be mainly pentaglutamates (Shin et al., 1972), a form which is not utilized by L. casei. Moreover, bovine liver conjugase has

been shown to produce exclusively monoglutamates (Silink et al., 1975). Therefore, if the hydrolysis of the polyglutamates was not complete within the three hour incubation time and/or if the HPLC procedure detects the pentaglutamates along with the monoglutamates, this would result in a large discrepancy between the HPLC and the microbial analyses. Another possible source of error could be the coelution of non-active compounds with the folate vitamers. This could be confirmed by collecting the eluting fractions and subjecting them to further analytical techniques.

A comparison between the effects of cooking on Total Folates without DHF and Total Folates with DHF reveals that the loss in the second is larger. Since the only difference between the two is the DHF content, it is clear that losses of the latter are reflected in Total Folates 2. However, in both cases, broiling appears to cause a slightly greater damage to the folates than frying. This was to be expected, since it took broiled samples longer to reach the internal temperature of 70°C. When the values for microbial assays are considered (Total Folates 3), a different perspective is observed. This is illustrated in the following comparison in which the percent loss in total folates activity due to the different treatments is depicted (Table 33).

Table 33. A comparison of Total Folate by three methods

	Total Folate 1 (HPLC - DHF)	Total Folate 2 (HPLC + DHF)	Total Folate 3 (Microbial)
B_1	45	68	41
B_2	55	78	40
F_1	45	64	52
F_2	52	73	49

According to the L. casei assay, frying had a more detrimental effect on the folate content of beef liver than broiling. While this finding is difficult to explain, the fact remains that the HPLC analysis and the microbial assay did not agree to a very great extent. A similar conclusion was made earlier by Gregory et al. (1982) on the folate content of cabbage.

The results for the effect of storage on the total folate content of beef liver, as measured by the L. casei method indicated that the folacin of raw liver in either vacuum or non-vacuum packages gradually decreased over time. In cooked liver on the other hand, it seemed to decrease for the first 30 days after which it stabilized. The HPLC analysis revealed different results. While a consistent decrease in both cooked and uncooked samples was observed over the first 60 days, a resurgence in the folate content of all samples was noticed at 90 days. This may be explained best by microbial activity which could have

induced the synthesis of the vitamin. Since no such effect was observed in vacuum packaged samples, it may be concluded that such microorganisms are probably aerobic in nature. Finally, as it would be expected, samples stored in vacuum, where less oxidative action occurred, gave higher folate values at all storage periods tested.

SUMMARY AND CONCLUSIONS

Borderline folate deficiency is being reported in various parts of the world. The primary population groups which are at risk are pregnant women, users of oral contraceptives, alcoholics and low-income female adolescents. The need for additional data on folacin activity in foods is imperative. The compilation of such data is hindered by the complex nature of dietary folates. Different biological activities and interconversions of the folate derivatives are suspected. Moreover, current folacin assays are subject to various interfering factors. Very few studies on folate dynamics have been reported in the literature. The studies which were reported usually involved aqueous buffers or liquid model food systems.

The purpose of the present research was to study the dynamics of folate behavior in an actual food system. Several processing and storage treatments were employed and their effect on the folate profile as compared to folate availability was established.

Two fresh beef livers were used in a randomized block design. Cooking, packaging and storage treatments were factorially arranged. Changes in four major folate derivatives and two main oxidation products were monitored by reverse phase HPLC technique. Total folate values were estimated by the Lactobacillus casei method and compared to those of the HPLC analysis.

The use of 2% ascorbate, nitrogen saturation and mild heat treatment during folate extraction resulted in high folacin values for beef liver. A peak corresponding to dihydrofolate was obtained in beef liver which would indicate the presence of dihydrofolate at levels previously unreported. The order of stability of the different liver folates towards cooking appeared to be: DHF < 5-CH_3-THF < 5-CHO-THF < THF. However, the stability of THF could have been an artifact of DHF reduction. L. casei assays revealed that frying had a more detrimental effect on folates than broiling. HPLC analysis indicated an opposite effect. Levels of THF, 5-CH_3-THF (and DHF) decreased during frozen storage while those of 5-CHO-THF generally increased. Microbial assays indicated smaller folate losses when liver was stored under vacuum. HPLC analysis revealed inconsistent results with respect to the changes in folates during storage. Folate interconversions and deconjugation of pteroylpolyglutamates could have contributed to these inconsistencies. The HPLC quantitation of total folates consistently resulted in higher folate values than the L. casei microbiological assays.

In view of these findings, current methodology for the extraction of folates from biological material might need to be reviewed. The use of a protective 2% ascorbate in the process could be beneficial, if not essential, for

folacin rich foods. Future work should be directed towards studying folate dynamics in actual food systems. Use of advanced analytical techniques and standardization of folate assay procedures should be investigated. The possible occurrence of dihydrofolate in beef liver or other food systems and its destruction by the current folate assay procedures should also be examined. Considerable nutritional implications may result because the ingestion of dihydrofolic acid has been shown to induce large increases in folate levels. Moreover, the adequacy of the L. casei assay, as a method of choice, in the assessment of folate availability should be re-evaluated. This assay technique could be yielding underestimates of the folacin value of food, resulting in a discrepancy between normal folate intakes and the folate RDA.

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APPENDIX

Appendix A. Stability of extract #1

Time (min)	pt-6-COOH	p-ABG	5-CH_3THF	THF	5-CHO-THF	DHF
0	2536	2450	433	1120	1541	8644
30	2430	2508	416	1082	1572	8555
60	2468	2567	412	1044	1596	8488
90	2508	2572	409	1065	1609	8445
Mean	2441	2524	418	1078	1580	8533
S.D.	64.7	57.4	10.8	32.2	29.9	86.7

Appendix B. Stability of extract #2

Time (min)	pt-6-COOH	p-ABG	5-CH_3THF	THF	5-CHO-THF	DHF
0	3543	3900	530	714	1288	17650
30	3530	3952	511	678	1303	16800
60	3582	4014	507	663	1333	16752
90	3641	4126	514	652	1352	16720
Mean	3574	3998	516	677	1319	16981
S.D.	49.8	97.2	10.1	27.0	28.9	447.5

All values are expressed as µg/100g on a dry basis

Appendix C. Pterin-6-Carboxylic acid

Cook	Package	Time (min)	I Control	I Treatment	II Control	II Treatment
Raw	Air	30	2640	3667	3480	4000
		60	3227	3173	3827	3333
		90	2320	2840	3493	4160
	Vacuum	30	2720	3080	3720	3427
		60	2213	2187	2793	3080
		90	1253	1813	3867	4460
Broiled	Air	30	2067	2478	1800	2156
		60	1433	2267	2456	2633
		90	1778	2111	2611	3133
	Vacuum	30	1156	1100	3578	2689
		60	1778	1387	2833	2100
		90	1211	1356	3067	3267
Fry	Air	30	1223	1015	3682	3165
		60	1106	1200	2718	2929
		90	1929	2871	1859	3329
	Vacuum	30	1294	1080	3188	2482
		60	1294	1449	3141	3365
		90	1247	1765	2282	3624

All values are expressed as µg/100g on a dry basis

Appendix D. p-aminobenzoyl glutamic acid

Cook	Package	Time (min)	I Control	I Treatment	II Control	II Treatment
Raw	Air	30	4013	2120	3107	1890
		60	1920	2200	3706	4722
		90	2480	3870	3933	6608
	Vacuum	30	3293	3616	3133	4240
		60	3053	3616	4146	5427
		90	3293	4273	3389	4573
Broiled	Air	30	2778	4311	4211	6824
		60	4511	6220	3744	5478
		90	3378	4898	3578	5344
	Vacuum	30	3444	2140	4078	2855
		60	4296	2378	4856	3205
		90	3711	4379	4611	5633
Fry	Air	30	3718	3112	5576	4294
		60	4188	4320	4965	5353
		90	3894	4592	4530	5659
	Vacuum	30	3965	2735	5577	4094
		60	3655	3771	4353	5318
		90	4072	5094	4488	6059

All values are expressed as µg/100g on a dry basis

Appendix E. Tetrahydrofolic acid

Cook	Package	Time (min)	I Control	I Treatment	II Control	II Treatment
Raw	Air	30	1627	520	1860	707
		60	814	584	1200	827
		90	1160	573	760	312
	Vacuum	30	1627	987	1267	760
		60	1453	693	1773	775
		90	1473	383	1200	360
Broiled	Air	30	1156	821	1622	1302
		60	1644	410	728	153
		90	1356	453	1100	446
	Vacuum	30	1211	778	535	367
		60	633	120	1267	256
		90	969	289	811	211
Fry	Air	30	1329	994	447	341
		60	1282	612	1011	506
		90	648	240	1048	450
	Vacuum	30	718	452	1037	612
		60	1024	481	506	192
		90	506	293	667	447

All values are expressed as µg/100g on a dry basis

Appendix F. 5-Methyl-tetrahydrfolic acid

Cook	Package	Time (min)	I		II	
			Control	Treatment	Control	Treatment
Raw	Air	30	440	147	520	152
		60	320	141	482	187
		90	408	400	493	458
	Vacuum	30	520	385	560	428
		60	493	375	560	393
		90	259	253	360	364
Broiled	Air	30	222	253	211	233
		60	167	100	178	115
		90	167	228	278	400
	Vacuum	30	167	124	211	148
		60	311	226	255	194
		90	129	90	167	111
Fry	Air	30	153	116	259	188
		60	224	129	294	176
		90	388	140	294	118
	Vacuum	30	200	210	241	241
		60	314	218	294	168
		90	224	204	241	241

All values are expressed as µg/100g on a dry basis

Appendix G. 5-Formyl-tetrahydrofolic acid

Cook	Package	Time (min)	I Control	I Treatment	II Control	II Treatment
Raw	Air	30	1733	2893	1770	3115
		60	1507	573	1720	577
		90	1533	2620	1280	2112
	Vacuum	30	1560	2106	2307	2947
		60	1218	1279	2200	2180
		90	1733	1880	2306	2630
Broiled	Air	30	600	1686	111	322
		60	178	40	533	111
		90	509	606	533	655
	Vacuum	30	211	293	533	744
		60	1089	244	422	111
		90	244	295	364	422
Fry	Air	30	1529	1024	1471	883
		60	635	922	565	788
		90	415	822	380	788
	Vacuum	30	1024	586	1565	472
		60	188	388	447	854
		90	258	1144	118	565

All values are expressed as µg/100g on a dry basis

Appendix H. "Dihydrofolic acid"

Cook	Package	Time (min)	I Control	I Treatment	II Control	II Treatment
Raw	Air	30	8103	6563	18086	14242
		60	8800	2893	21370	8102
		90	8693	8986	17650	18225
	Vacuum	30	10146	11400	16093	16756
		60	10146	9853	22713	20326
		90	8954	8506	16233	14850
Broiled	Air	30	2178	1807	3967	3656
		60	2733	2214	4122	2927
		90	2256	1208	4922	2700
	Vacuum	30	2578	2527	4600	4289
		60	3789	1767	3178	1422
		90	1695	1932	3700	4354
Fry	Air	30	2647	1456	5377	3280
		60	2047	1003	5882	3188
		90	2605	2894	5200	5133
	Vacuum	30	2470	1106	4356	2188
		60	3390	2306	4541	2859
		90	2783	2894	4365	4706

All values are expressed as µg/100g on a dry basis

Appendix I. Total folates by HPLC. DHF not included

Cook	Package	Time (min)	I Control	I Treatment	II Control	II Treatment
Raw	Air	30	3800	3560	4150	4014
		60	2641	1298	3402	1591
		90	3101	3593	2533	2882
	Vacuum	30	3703	3478	4134	4135
		60	3164	2347	4533	3348
		90	3465	2516	4066	3354
Broiled	Air	30	1978	2760	2014	1857
		60	1989	550	1439	379
		90	2032	1287	1921	1501
	Vacuum	30	1589	1195	1279	2760
		60	2033	590	1944	561
		90	1340	674	1342	744
Fry	Air	30	3011	2134	2177	1412
		60	2141	1663	1870	1470
		90	1451	1202	1722	1356
	Vacuum	30	1942	1248	2843	1325
		60	1526	1087	1247	1214
		90	988	641	1026	1253

All values are expressed as µg/100g on a dry basis

Appendix J. Total folates by HPLC. DHF included

Cook	Package	Time (min)	I Control	I Treatment	II Control	II Treatment
Raw	Air	30	11903	10123	22236	18216
		60	11441	4191	24772	9711
		90	11794	12579	20183	21107
	Vacuum	30	13853	14878	20227	20891
		60	13310	12200	27246	23674
		90	12419	11022	20099	18204
Broiled	Air	30	4156	4567	5981	5513
		60	4722	2764	5561	3306
		90	4288	2555	6843	4201
	Vacuum	30	4167	3722	5879	5548
		60	5822	2357	5122	1983
		90	3035	2606	5042	5098
Fry	Air	30	5658	3590	7554	4692
		60	4188	2660	7752	4658
		90	4056	4096	6922	6489
	Vacuum	30	4412	2354	7208	3513
		60	4916	3393	5788	4073
		90	3771	4535	5391	5959

All values are expressed as µg/100g on a dry basis

Appendix K. *L. casei* assay: Total folates

Cook	Package	Time (min)	I Control	I Treatment	II Control	II Treatment
Raw	Air	30	1020	557	1067	527
		60	920	360	1300	580
		90	960	413	933	410
	Vacuum	30	1077	950	1037	880
		60	1050	567	1377	673
		90	993	543	1100	573
Broiled	Air	30	583	367	600	350
		60	614	186	767	256
		90	661	225	658	206
	Vacuum	30	564	472	617	494
		60	600	469	692	547
		90	517	408	744	583
Fry	Air	30	474	209	574	285
		60	506	209	532	215
		90	459	209	603	247
	Vacuum	30	476	406	471	391
		60	444	300	479	315
		90	532	341	447	274

All values are expressed as µg/100g on a dry basis

VITA

Fadi M. Aramouni was born on March 20, 1956 in Beirut, Lebanon. He attended the College Notre-Dame de Jamhour High School where he graduated in June 1973. His college work started at the School of Arts and Sciences of the American University of Beirut where he received his Bachelor of Science degree in Biology-Chemistry in September 1977. This was followed by graduate studies at the Faculty of Agriculture and Food Science of the American University of Beirut where he was awarded the Master of Science degree in Food Technology in September 1980.

After working 26 months for the Industrial Training Division of the Arabian American Oil Company in Dhahran, Saudi Arabia, he came to the United States to pursue his doctoral studies. In January 1983, he enrolled in the Department of Food Science of the Louisiana State University where he is at present a candidate for the Doctor of Philosophy degree.

DOCTORAL EXAMINATION AND DISSERTATION REPORT

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Major Field: Food Science

Title of Dissertation: Dynamics and Bioavailability of Folates in Bovine Liver as a Function of Processing and Frozen Storage

Approved:

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